Helsingin yliopisto
Elintarvike- ja ravitsemustieteiden osasto

Department of Food and Nutrition
University of Helsinki

EKT-sarja 1911
EKT series 1911

Physical and oxidative stability of modified faba bean protein isolate (FBPI) in oil-in-water emulsions

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Academic Dissertation

To be presented for public examination with the permission of the Faculty of Agricultural and Forestry of University of Helsinki, in Walter Hall, EE building in Viikki, on the 13th of March, 2020, at 12 o'clock noon.

Helsinki, 2020

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ISBN 978-951-51-5804-8 (paperback)
ISBN 978-951-51-5805-5 (PDF; http://ethesis.helsinki.fi)
ISSN 0355-1180

Unigrafia
Helsinki 2020

Abstract

Faba bean is a legume that is not only rich in proteins but also is well adapted to the short growing seasons in the Nordic and Baltic countries. Considering the economic cost and sustainability issues, growing interests are focused on plant proteins. In this study the potential applications of faba bean protein isolate (FBPI) and modified FBPIs in oil-in-water (O/W) emulsions were investigated. The overall aim of the research was to study how different modifications of FBPI affected the physical and oxidative stability of O/W emulsions.

Modification of FBPI by microbial transglutaminase (MTG) induced protein cross-linking. MTG treatments raised the protein net surface charges by 5-8%, and increased the emulsion particle size by 19-135%. MTG treatments for longer time (120 and 240 min) induced protein oxidation and excessive surface hydrophobicity, resulting in decreased emulsifying activity and physical stability of emulsions. On the other hand, a short time (60 min) MTG treatment improved FBPI's potential to maintain the physical stability while improving lipid oxidative stability of the emulsion. This might be attributed to thicker interfacial layer, larger droplet size, and protective effect of protein.

Alcalase hydrolysis of FBPI to a degree of hydrolysis (DH) of 4% produced an emulsion with improved physical stability and least of lipid oxidation while maintaining protein oxidative stability as compared to emulsions prepared with native and extensively hydrolyzed (DHs of 9 and 15%) FBPIs. FBPI hydrolysates with DH of 4% exhibited molecular weight better applicable to interfacial layer stability, increased surface net charge for more repulsive electrostatic force, and increased hydrophobicity.

Considering the important role of interfacial layer thickness in emulsion stability, FBPI and chitosan (CH) were associated in different ways to construct different types of interfacial layer. Emulsions with layer-by-layer (LBL) interfacial structure not only showed better physical stability, but also had superior oxidative stability. This might be attributed to that CH assisted in forming LBL interface, which increased the interfacial layer thickness/compactness and maintained the interfacial protein adsorption.

In conclusion, the emulsifying functionality of FBPI, and thereby the stability of FBPI containing O/W emulsions can be improved by different ways of modifications via several mechanisms.

Acknowledgements

The thesis was carried out at the Department of Food and Nutrition of the University of Helsinki. The work was financially funded by the China Scholarship Council and the Finnish Food Research Foundation. I gratefully appreciate their financial supports.

First of all, my sincerely gratitude to my supervisor Prof. Marina Heinonen. I would like to thank you for giving me the opportunity to study at the University of Helsinki as a master student, and then accepting me as a PhD student in your research group. I especially appreciate your insightful advice and caring mentoring, not only on science but also on my life as well. With your continuous support and encouragement, I have got what I have been looking for in both academia and my personal life. You really have a magical and charming smile which makes me inspired and warm.

I am grateful to Assistant Prof. Kirsi S. Mikkonen and Doc. Tuula Sontag-Strohm for participating in my follow-up group during my PhD work. Thank you all for the useful comments and technical supports. I sincerely thank Prof. Vieno Piironen, for your guidance and leadership during the past years in food chemistry.

My deep gratitude also goes to Doc. Riitta Partanen and Prof. Karin Schwarz for the pre-examination of this dissertation. I sincerely appreciate your valuable time and constructive comments, which help me to finalize my dissertation smoothly.

I would like to express my friendly gratitude to my collaborators and co-authors: Prof. Srinivasan Damodaran, Mamata Bhattarai, Assistant Prof. Kirsi S. Mikkonen, Dr. Ruisong Pei and Doc. Leena Peltonen. They unselfishly provided facilities, knowledge, and skills for my experiments and manuscript compiling. Further, I thank all the research and teaching staffs who were always happy to share their knowledge and technical support patiently: Doc. Anna-Maija Lampi, Doc. Fred Stoddard, Dr. Göker Gürbüzand, Dr. Marjo Pulkkinen, and Dr. Fabio Valoppi. Thank you also to technical staffs Taru Rautavesi, Mikko Kan-Gas, Anu Riikka Heiman-Lindh, Miikka Olin, and Outi Brinck. Without your help, I would not able to complete my work efficiently. As I finished my master degree here as well, I would like to use this opportunity to express my thanks to teachers who open my eyes in food science: Dr. Per Ertbjerg, Doc. Susanna Kariluoto, Doc. Ndegwa Maina, Doc. Velimatti Ollilainen, and Doc. Pekka Varmanen.

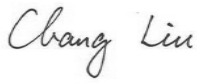
I would like to thank my colleagues and friends Dr. Zhen Yang, Bei Wang, Yuetuan Zhang, Fengyuan Liu, Xiaocui Han and Dr. Yan Xu, for the lively and friendly atmosphere. A

special thank-you is directed to my cousin Kun Qian and my friend Xiao Han, I appreciate your trust and support. I wish you all the best in the future.

My deepest gratitude goes to my dearest parents： 谢谢妈妈和爸爸，是你们的付出成就了我的今天。妈妈，对我来说你是全天下最伟大的妈妈，是你用无条件的支持和无私的爱，在帮助我实现一件件的梦想，谢谢您。同时，也要感谢我的婆婆和公公，谢谢你们在背后默默的支持着我完成学业。

To my husband, words cannot describe my appreciation and gratitude for all you have done for me. Thank you for being my best friend, and loving me. Although you are not always by my side during the past years, I always feel you in my heart as you do.

Helsinki, Oct 2019



Chang Liu

List of original publications

This thesis is based on the following papers:

I. **Liu C**, Damodaran S, Heinonen M. 2019. Effects of microbial transglutaminase treatment on physiochemical properties and emulsifying functionality of faba bean protein isolate. LWT-Food Sci Technol 99: 396-403.

II. **Liu C**, Bhattarai M, Mikkonen KS, Heinonen M. 2019. Effects of enzymatic hydrolysis of fava bean protein isolate by Alcalase on the physical and oxidative stability of oil-in-water emulsions. J Agric Food Chem 67(23): 6625-6632.

III. **Liu C**, Pei R, Peltonen L, Heinonen M. Assembling of the interfacial layer affects the physical and oxidative stability of faba bean protein-stabilized oil-in-water emulsions with chitosan. Food Hydrocoll 102.

Contribution of the author to the papers I to III:

I. Chang Liu planned the study together with the other authors, and carried out all the experiments. She was responsible for interpreting the results, preparing the manuscript and served as the corresponding author for the paper.

II. Chang Liu planned the study together with Prof. M. Heinonen. She was responsible for all the experimental work. She had the main responsibility for interpreting the results, preparing the manuscript and served as the corresponding author of the paper.

III. Chang Liu designed the experimental work together with Prof. M. Heinonen. She performed all experiments and had primary responsibility for result interpretation. She prepared the manuscript and served as the corresponding author.

Abbreviations

ANS	8-anilino-1-naphthalenesulfonic acid
BS	backscattering
BSA	bovine serum albumin
CDs	conjugated dienes
CH	chitosan
CLSM	confocal laser scanning microscope
DH	degree of hydrolysis
DH0-FBPI	FBPI dispersion with inactive Alcalase
DH4/9/15-FBPI	FBPI dispersions treated by Alcalase to DH of 4/9/15 %
DH0/4/9/15	emulsions prepared with DH0/4/9/15-FBPI
DNPH	2,4-dinitrophenylhydrazine
DTNB	5,5'-dithio-2-nitrobenzoate
$d_{3,2}$	surface mean diameter
$d_{4,3}$	volume mean diameter
EAI	emulsifying activity index
EMC	emulsions emulsified by soluble FBPI-CH complex
EMFB	emulsions emulsified by FBPI
EML	emulsions first emulsified by FBPI, and then CH as a second layer
EMN	emulsions emulsified by FBPI, with free CH in the aqueous phase
F_{ads}	protein adsorption fractions
FBPI	faba bean protein isolate
FI	fluorescence intensity
FITC	fluorescein isothiocyanate
GC-MS	gas chromatography-mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
MTG	microbial transglutaminase
MTG0-FBPI	FBPI dispersion with inactive MTG
MTG60/120/240-FBPI	FBPI dispersions with 60/120/240 min MTG treatment
MTG0/60/120/240	emulsions prepared with MTG0/60/120/240-FBPI
MW	molecular weight
O/W	oil-in-water

O/W/O	oil-in-water-in-oil
pI	isoelectric point
P-O/W EM	protein emulsified oil-in-water emulsion
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPB	sodium phosphate buffer
TCA	trichloroacetic acid
TSI	Turbiscan stability index
W/O	water-in-oil
W/O/W	water-in-oil-in-water
λ_{max}	maxima of fluorescence emission
Γ_s	surface load

For a list of the amino acids and their abbreviations with three letters see Table 1 on page 14.

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1 Introduction

As one common form of food, the oil-in-water (O/W) emulsion is a thermodynamic-unstable system and is highly susceptible to oxidation due to its large interfacial area outside oil droplets (Elwell et al. 2004). Proteins are commonly added to O/W emulsions not only as emulsifiers, but also as nutrients. Instability of food emulsions causes undesirable sensory outcomes and nutrition loss, and therefore becomes a major concern of researchers, food manufactures, and customers. The physical instability and lipid oxidation can be perceived readily due to visible structural alterations, rancidity, or color change. Thus, when evaluating the stability of emulsions, usually either only physical stability or together with lipid oxidation is considered. However, in protein-contained O/W emulsions, proteins oxidation should not be neglected. In fact, protein oxidation not only affects food quality and nutritional values, but also plays important roles in lipid oxidation; therefore, it should not be underestimated (Estevez and Luna 2017). By now, only a few studies considered protein oxidation when evaluating emulsion stabilities (Ogawa et al. 2003; Shao and Tang 2014; Qiu et al. 2015). Therefore, there is a need for comprehensive investigations of physical and lipid oxidative stabilities, as well as protein oxidative stability when assessing the emulsion stability.

Growing research interests have been focused on plant proteins as substitutes for animal proteins, due to the economic cost and sustainability issues (Multari et al. 2015). Faba bean contains high level of proteins (average of ~32% *vs.* average of ~ 29% in other grain legumes), which makes it a promising and desirable protein source. It also has excellent adaptability in a wide range of climates and environmental conditions (e.g. cold climate and dry land) worldwide (Lizarazo et al. 2015). Meanwhile, faba bean is considered as a sustainable crop in that it offers ecosystem services such as renewable inputs of nitrogen into crops and soil via biological nitrogen fixation and diversifications of cropping systems (Lizarazo et al. 2015). Therefore, faba bean can meet the increasing demands for protein in an environmentally friendly way.

Recently, the native faba bean protein has been studied as a natural emulsifier in O/W emulsions (Gürbüz et al. 2018b). However, the native faba bean protein exhibited unfavorable performance on emulsifying capacity and emulsion stability, which could be attributed to its intrinsic physiochemical properties (e.g. surface charge, hydrophobicity, and molecular weight) that are decisive factors for emulsifying properties (Bigelow 1967; Schwenke 2001b). As shown in many studies, emulsifying properties of a protein can be

improved by various physical, chemical, and enzymatic modifications. The majority of these efforts focused on modifying the configuration of the protein, changing the surface charge or hydrophobicity of the protein, increasing the layer thickness of the interfacial layer, altering the conformation of the oil-water interface, etc. (Chen et al. 2011c; Lundblad 2014; Luisa et al. 2015). So far, only a few studies reported that heating, high-pressure treatment, and cross-linking might improve emulsifying property of faba bean protein isolate (FBPI) (Galazka et al. 1999; Johnston et al. 2015). However, these studies generally lacked consideration of how the modifications affected the overall oxidative stability of emulsions and the underlying mechanisms. Also, studies on other types of modifications (e.g. enzymatic hydrolysis, combination with polysaccharides) on FBPI are rare.

This dissertation presents an overview of published literature on the stability of protein-emulsified O/W emulsions, as well as protein modifications for improving emulsifying property of FBPI and oxidative stability of corresponding emulsions. The goal of this study is to identify suitable modifications of FBPI to improve the physical and oxidative stability of O/W emulsions. Meanwhile, focus was placed on the interfacial structure of oil droplets in emulsions to reveal the role of interfacial layer on oxidative stability of emulsions. Furthermore, the relationship between physical stability, lipid and protein oxidation in O/W emulsions was explored.

2 Review of the literature

2.1 Faba bean proteins

2.1.1 Protein composition

The protein ingredient market is shifting away from animal-derived and soybean-derived proteins toward other legume-based alternatives because of their low cost, environmental adaptability, nutrition value, low amount of allergens, and optimum product diversity (Martinez et al. 2016). Faba bean protein is a promising alternative protein source to fulfill the market demands because of its nutritional value and many functional properties.

Faba bean storage proteins mainly consist of legumin-like globulins (11S), vicilin-like globulins (7S), and albumins (2S) (Hossain and Mortuza 2006). Unlike cereals, the prolamin and glutelin fractions are present in very low proportions and have consequently been rarely studied. Legumin-like globulins (11S) are hexameric proteins containing six intermediary subunits - αβ (Bailey and Boulter 1970), with a high molecular weight (MW) of 300-400 kDa. Each intermediary subunit comprises an acidic α subunit (α1, α2, α3, α4) that has an average MW of ~ 36 kDa and a basic β subunit (β1, β2, β3, β4) that has an average MW of ~ 22 kDa (Wright and Boulter 1974). Within legumin-like globulins, α subunits are hydrophilic and situated at the surface of the molecule, whereas β subunits contain more hydrophobic amino acids (e.g. Leucine (Leu), Valine (Val), and Phenylalanine (Phe)) hidden in the core to reduce the possibility of contacting with water. It is likely that α and β subunits are mainly connected by a single disulfide bond existed in the legumin molecule itself. Besides, the intermediary subunits are also closely packed via electrostatic and hydrophobic interactions to form a stabilized rigid structure. Thus, the intermediary subunits in faba bean 11S protein are heterogeneous and minor differences are observed in various genotypes, depending on which α and β subunits are combined (Wright and Boulter 1974). Classically, vicilin-like globulin (7S) is a globulin remaining soluble at pH 4.7 (Wright 1973). In faba bean proteins, it is a trimeric protein containing three subunits, with an average MW of 150 ± 2.5 kDa. When vicilin extracted from mature faba beans were electrophoresed on either reduced or non-reduced sodium dodecyl sulfate (SDS) gels, it yielded the same five major bands with MWs of 55.5, 46.0, 43.4, 33.3, and 31.5 kDa. This indicates that sulphydryl groups, if present, play no role in the bonding between subunits. The ratios of vicilin subunits change during seed development. In the mature faba beans, the vicilin fraction accounts for only ~ 20% of the total globulins (Bailey and Boulter 1972; Wright and Boulter 1974). Albumins (2S) in faba bean constitute ~ 10% of the protein, which are much less than

globulins that represent more than 70%. Thereby, the albumins from faba bean proteins have been investigated far less extensively than the globulins. The MWs of albumins are much more variable than globulins, ranging from 10 to 100 kDa. The albumins contain more Lysine (Lys), Val, Threonine (Thr), Methionine (Met), and Tryptophan (Trp), but less Arginine (Arg), Leu, and Phe than the globulins (Bhatty 1982).

According to Kimura et al. (2008), the thermal denaturation midpoint temperatures of 11S and 7S globulins in faba bean are higher than those in soybean (95.4 °C *vs.* 93.5 °C for 11S, and 83.8 °C *vs.* 78.5 °C for 7S) at ionic strength of 0.5, which indicates that faba bean protein exhibits better thermal stability than soybean protein and is more suitable for the production of foods requiring high thermal stabilities. The maxima of fluorescence emission (λ_{max}) for legumin-like globulins (11S), vicilin-like globulins (7S), and albumins (2S) are 320 nm, 329 nm, and 338 nm, respectively. Zhao et al. (2011) deduced that 11S, 7S globulins, and 2S albumins are blue-shifted from Trp. The shifts in the λ_{max} of emission spectra of faba bean proteins from pure Trp (~ 350nm) may be caused by protein conformational transition, subunit association, ligand binding, or denaturation. The deviation of λ_{max} in 11S (320 nm) from Trp (350 nm) suggests that Trp residues in 11S are buried in the hydrophobic core of protein. The λ_{max} in 2S (338 nm) is closer to that in Trp, suggesting that more Trp residues are located on the surface of 2S compared to 11S.

The globulin/albumin (G/A) or legumin/vicilin (L/V) ratio of faba bean protein is varied depending on various intrinsic (e.g. seed coat type, flower color, seed color, and seed size) and extrinsic factors (e.g. agronomic factors and extraction methods). Generally, aqueous extraction followed by isoelectric precipitation is the widely used technique for producing legume protein isolates (Singhal et al. 2016), and the produced protein isolates mainly contain legumin-like globulin and vicilin-like globulin. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Wright and Boulter (1974) identified several main bands with MWs of ~37, ~24, and ~21 as the α- and β-subunits of legumine-like 11S globulins and vicilin-like 7S globulins, respectively. Compared to the raw flour, the extraction process for FBPI eliminated only several faint bands which were presumed to be albumin storage proteins (Singhal et al. 2016). It is noteworthy that FBPI is virtually free of the glucosides vicine and convicine, which are anti-nutritional compounds that can induce favism in individuals with low levels of erythrocyte glucose 6-phosphate dehydrogenase (Vioque et al. 2012; Pulkkinen et al. 2015). However, the use of FBPI is very limited, partially because of the poor understanding of its food functionalities.

2.1.2 Amino acid composition of FBPI

The amino acid composition of FBPI is similar to other grain legumes, and is characterized by good nutritional quality (Vioque et al. 2012). In addition, the measurement of standardized ileal digestibility showed that there was no significant difference between faba beans and soya beans (O'Neill et al. 2012). The protein amino acid composition of faba bean flour and FBPI are summarized in **Table 1** (Vioque et al. 2012). FBPI contains large amounts of hydrophilic Arg, Aspartic acid (Asp), and Glutamic acid (Glu) which all together account for almost 50% of total amino acids. Leu and Lys contents are significantly higher than other amino acids. Sulfur amino acids in FBPI are relatively deficient, with Met as the only limiting amino acid in FBPI. However, the deficiency can be overcame by incorporating cereals which contain substantially higher Met and Cysteine (Cys) in the diets (Chatterjee and Abrol 1975). It is worth noting that the FBPI has similar amino acid profile with its raw flour, but is more deficient in Met and Cys. This is probably due to loss of sulfur amino acids-rich albumins during extraction (Vioque et al. 2012).

Table 1. Amino acid composition of *Vicia* faba flour and faba protein isolate (Mean ± SD).

Amino acid	Flour (%)	Protein isolate (%)
Asp + Asn	12.4 ± 0.1	13.3 ± 0.1
Glu + Gln	18.6 ± 0.0	19.9 ± 0.0
Ser	6.4 ± 0.0	6.3 ± 0.0
His	2.8 ± 0.0	2.8 ± 0.0
Gly	5.9 ± 0.0	4.9 ± 0.0
Thr	4.7 ± 0.0	3.7 ± 0.0
Arg	9.8 ± 0.1	10.0 ± 0.0
Ala	5.3 ± 0.0	4.4 ± 0.0
Pro	1.6 ± 0.2	3.4 ± 0.2
Tyr	2.6 ± 0.0	2.6 ± 0.0
Val	4.2 ± 0.1	4.1 ± 0.0
Met	0.2 ± 0.0	0.1 ± 0.0
Cys	1.0 ± 0.0	0.5 ± 0.0
Ile	3.9 ± 0.0	3.8 ± 0.0
Trp	0.4 ± 0.0	0.3 ± 0.1
Leu	7.8 ± 0.0	8.0 ± 0.1
Phe	4.7 ± 0.0	4.9 ± 0.0
Lys	7.7 ± 0.0	7.0 ± 0.0

Compiled from Vioque et al. (2012)

2.1.3 Emulsifying property of FBPI

Protein functional properties are defined as the physical and chemical properties which affect the behaviors of proteins in food systems (Kinsella 1982). Solubility, water binding, fat binding, emulsification, foaming, gelation, thickening, and flavor binding are the most interesting functional properties of proteins. Of these functional properties, the emulsifying property is particularly important for O/W emulsions which are common structures in food products. The emulsifying property of a protein is greatly influenced by its structure, molecular size, surface hydrophobicity, net charge, molecular flexibility on the interfacial layer, as well as processing conditions such as pH, temperature, and the interactions that occur between proteins and other food components (Boye et al. 2010).

Protein solubility is important for the emulsification by facilitating the migration of proteins to and spreading at the oil-water interface (Wu et al. 1998). With an isoelectric point (pI) of ~ 4.5, the solubility of FBPI markedly decreases at near pI (~ 4 to 6) and mostly solubilizes at pH of 8-9 (Otegui et al. 1997). As reported by several studies, the solubility of FBPI is typically between 80-85 % at pH 7.0 (Carbonaro et al. 1997; Johnston et al. 2015). pH is a decisive factor for solubility, as it further affects hydrophobicity and surface charge of proteins which influences the equilibrium between the protein-solvent (hydrophilic) and the protein-protein (hydrophobic) interaction, and electrostatic repulsion, respectively. Generally, proteins with lower surface hydrophobicity tend to exhibit higher solubility (Bigelow 1967), and proteins with higher net charges tend to foster sufficient electrostatic repulsion between proteins to overcome attractive forces and retain higher solubility in solution (Johnston et al. 2015). In addition, MWs of proteins influence solubility as well. FBPI hydrolysates with smaller MWs exhibited higher solubility because smaller peptides produced by hydrolysis can form stronger hydrogen bonds with water and become more soluble (Xu et al. 2016).

Surface hydrophobicity and net surface charge of proteins are proposed to be the most important factors for the emulsification process in a protein-emulsified O/W emulsion (P-O/W EM) (Schwenke 2001a). The amphiphilic nature of proteins allows them to remain in the aqueous phase while simultaneously adsorb at the surface of oil droplets where proteins generate stabilizing electrostatic forces and steric interactions (Claesson et al. 2001). Nakai and colleagues (1980) found a significant correlation between hydrophobicity and emulsifying properties. The hydrophobic patches of proteins, which relate to the hydrophobic amino acid residues exposed at the surface, are the precondition for protein adsorption at the oil-water interface (Schwenke 2001a). Johnston et al. (2015) reported the

decreasing hydrophobicity with: chickpea > lentil = soya > faba bean protein isolates. It seems that FBPI would show he poorest emulsifying capacity based on its lowest surface hydrophobicity relative to the other protein isolates. In fact, emulsification property of proteins is more dependent on the balance between hydrophobicity and hydrophilicity which determines accessible surface area, unfolding, and re-orientation at the interface (Schwenke 2001b). In addition, proteins with higher net charge are more likely to form stable emulsion. Having a high protein surface charge not only promotes greater hydration of proteins (Schwenke 2001b), but also induces high repulsive interfacial charges. This repulsive interaction is one of the forces governing stability of emulsion. Therefore, higher value of surface charge and resultant higher electrostatic repulsion would imply a more stable P-O/W EM to inhibit coalescence and flocculation (Li and Tian 2002). The FBPI carries a net negative charge at pH above it pI and positive charge at pH below. The net negative charge, for example at pH 7.0, is mainly due to the negatively charged side chains of Asp (pKa = 3.65) and Glu (pKa = 4.25) amino acids spatially located on the protein surface (Lide 2004). Thus, the net charge of FBPI in emulsions is primarily dependent on pH and other components at the interface.

Interfacial tension is another important factor for emulsifications. In the preparation of P-O/W EMs, proteins migrate to the oil-water interfaces where they re-align themselves to position their surface hydrophobic amino acids toward the oil phase and hydrophilic moieties within the aqueous phase. Then, proteins form an interfacial film surrounding oil droplets that maintains stability through electrostatic repulsive forces and steric stabilization. During this process, proteins align at the interface, decrease the interfacial tension, and facilitate formation of stable P-O/W EMs (Singhal et al. 2016). Although mostly proteins are not as effective as low MW surfactants such as phospholipids in reducing the interfacial tension, some proteins (e.g. soybean, pea, egg, whey, and wheat) could still form stable emulsions due to their adequate surface activity (Singhal et al. 2016). It has been demonstrated that FBPIs can decrease interfacial tension efficiently, thus working as emulsifiers (Johnston et al. 2015). The ability to reduce interfacial tension closely relates to the FBPI's conformation and its ability to unfold at interfaces (Van Vliet et al. 2002).

2.2 Emulsions and their stability

2.2.1 Emulsions

Emulsions exist both as common foods (e.g. milk) or constitute a structure in food products to provide specific properties. Emulsions also affect various important physicochemical and organoleptic characteristics such as appearance, flavor, texture, taste, and shelf life. Emulsions are colloidal systems that consist of two immiscible liquids, where one of the liquids is dispersed as spherical droplets in the other liquid. In most of emulsion-type foods, the two immiscible liquids refer to oil and water. Depending on the relative spatial distribution of the two phases, emulsions are classified as either O/W emulsions in which oil droplets disperse in a water phase, or water-in-oil (W/O) emulsions in which water droplets disperse in an oil phase. In addition, there are other types of emulsions, such as oil-in-water-in-oil (O/W/O) emulsions and water-in-oil-in-water (W/O/W) emulsions (McClements 2015; Bakry et al. 2016) (**Figure 1**).

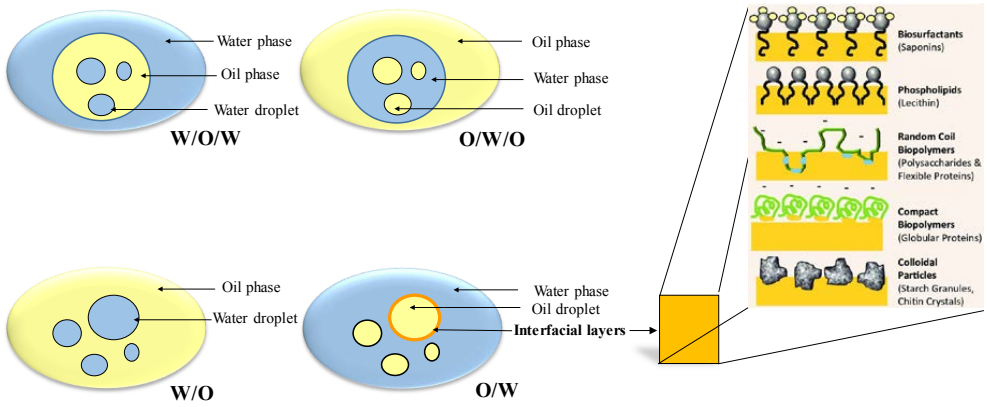

Figure 1. Four normal emulsion systems, and common natural emulsifiers applied in an O/W emulsion including biosurfactants, phospholipids, biopolymers, and colloidal particles. (Compiled from Bakry et al. 2015, and McClements and Gumus 2016)

For large-scale manufacturing in food industry, O/W emulsions with small droplets are usually made by homogenizing oil and aqueous phase using high-energy mechanical devices such as high-speed blenders and high-pressure valve homogenizers (Tesch et al. 2003). Despite the thorough homogenization, an O/W emulsion usually rapidly separates into a layer of oil on top of a layer of water in the absence of emulsifiers. This is because emulsions are thermodynamically unstable. The contact between oil and water is thermodynamically unfavorable as oil droplets tend to merge with each other (Elwell et al. 2004). Thereby, the use of emulsifiers is common in the manufacturing of food products. Emulsifiers are surface-

active compounds that facilitate the homogenization process and maintain emulsion stability. Emulsifiers can reduce the interfacial tension between oil and water phase, and consequently decrease the energy needed during homogenization. In addition, emulsifiers can easily adsorb to the surface of freshly formed oil droplets due to the amphiphilic nature, and consequently form a protective layer that prevents oil droplets from aggregation (Whitehurst 2004). Meanwhile, emulsifiers give the food an appealing appearance, freshness, and good quality which attract consumers. There are many types of emulsifiers that are widely used in food industry including synthetic, semi-synthetic, and natural emulsifiers (McClements and Gumus 2016) (**Figure 1**). In food industry, the most commonly used emulsifiers are lecithin (E 322), and mono- and di-glycerides of fatty acids (E 471). Lecithin (E 322) is naturally present in cell membranes. Currently, the commercial lecithin usually consists of a mixture of various phospholipids that are obtained mostly from soybean oil. Because of the EU-requirement about allergens and genetically modified crops, a gradual shift to other source (e.g., sunflower oil) is taking place. Mono- and di-glycerides of fatty acids (E 471) are produced from glycerol and natural fatty acids that are mainly from animal fat or vegetable oil. Although mono- and di-glycerides are classified as safe emulsifiers with E-code, they still arouse consumer concern occasionally as they contain small amounts of trans fats. Sodium caseinate (E 469), an emulsifier originally from milk protein casein rather than from animal fat or vegetable oil, has favorable emulsification and stabilization functions. However, vegans and vegetarians may want to avoid ingredients from animal sources (Whitehurst 2004). Increasing awareness about human and environmental health is encouraging food manufacturers to switch food ingredients to more natural and plant-based materials. Plant-proteins are increasingly studied and employed as emulsifiers, including but not limited to chickpea, corn, lentil, peas, lupin, and soybean protein (Johnston et al. 2015; McClements et al. 2017). Some native plant proteins have good potentials as natural emulsifiers for a P-O/W emulsion, but in other cases they need to be modified (physically, chemically, or enzymatically) to enhance their functionality. To evaluate whether a plant protein could serve as a good emulsifier, both the physical and chemical stability of formed emulsions should be considered.

2.2.2 Physical stability of O/W emulsions

Physical stability refers to the ability of an O/W emulsion to resist changes in the spatial distribution or structural organization over time. Creaming, flocculation, and coalescence are the main examples of physical instability within emulsions (McClements 2015). Identifying the mechanisms behind those instabilities would help to effectively control the

stability of an emulsion. In this section, the physical basis, methods for controlling, and measurements regarding physical stability will be explained.

2.2.2.1 Creaming

Creaming is one of the most common examples of instability in O/W emulsions. In an O/W emulsion, oil droplets have a lower density than the aqueous phase, and consequently tend to move upward and accumulate at the top (Robins 2000). As shown in **Figure 2A**, larger oil droplets move faster than smaller ones. In the end, a creamed layer of oil droplets is floating on the top, leaving a clear aqueous phase at the bottom. The final thickness of formed creamed layer is determined by both oil concentration and how the oil droplets are packed. Creaming leads to various undesirable qualities. For example, creaming induces separation of oil layer on the top and aqueous layer at the bottom, which makes the food products less visually appealing to customers. In addition, the inhomogeneity in texture would have negative taste and mouthfeel. More importantly, creaming can lead to further instability such as flocculation and coalescence, which is discussed below (McClements 2015).

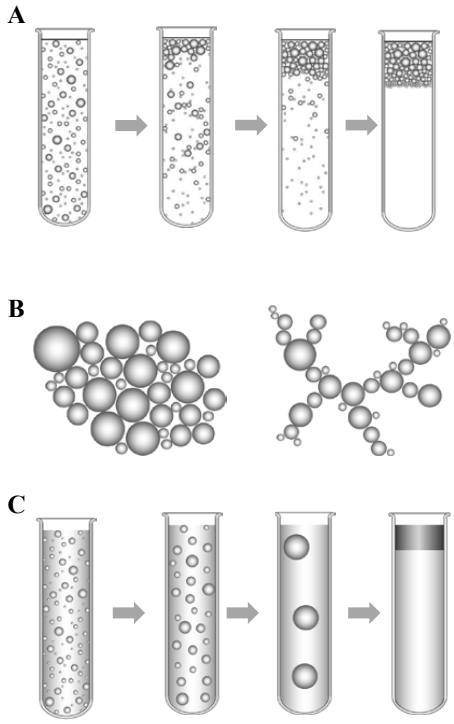

Figure 2. Schematic representations of physical stability of O/W emulsions. (A) Time-dependent creaming; (B) schematic representation of flocculation with close packing (left), and flocculation with open packing (right); (C) time-dependent droplet coalescence with complete separation of the oil and aqueous phase (Adapted from McClements 2015).

Considering the adverse effects and the underlying mechanisms of creaming, there are several ways to reduce the occurrence of creaming. The first way is to minimize the difference of density between oil and aqueous phase which is the primary driving force for creaming. This might be achieved by adding "weighting agents" to oil phase to match the density of water before homogenization (Piorkowski and McClements 2014). The density difference might also be minimized by using emulsifiers which can form a relative thick and dense interfacial layer (Piorkowski and McClements 2014). Another way to reduce creaming is to reduce oil droplet size through homogenization, as the rate of creaming is proportional to the droplet size (Dickinson 1992). Lastly, increasing the oil concentration helps to inhibit creaming. This is because oil droplets are restricted within the emulsion due to the high volume (McClements 2012; Akoh 2017). Common methods for accessing creaming include measuring the height of the serum layer over time, and measuring the percentage of transmitted and/or scattered light as a function of emulsion height by a vertical-scanning light scattering method (Mengual et al. 1999; McClements 2015).

2.2.2.2 Flocculation

Flocculation is generally defined as the aggregation of droplets without coalescence occurring (McClements 2015). In general, flocculation occurs when two or more oil droplets come in close proximity but still maintain individual integrity (**Figure 2B**). Flocculation can decrease emulsion quality in that it not only accelerates creaming due to increased particle size, but also can increase the emulsion viscosity or even lead to undesirable formation of gel (McClements 2015). Therefore, controlling flocculation is critical for maintaining emulsion quality.

Several strategies have been developed to reduce flocculation in emulsions. Because creaming and mechanical agitation are major causes of flocculation, reducing the creaming process by minimizing density difference and avoiding mechanical agitation are commonly used methods (McClements 2015). Another important strategy is to increase the repulsive interactions (e.g. electrostatic repulsion and steric hindrance) between oil droplets to a significantly higher level than the attractive interactions (McClements 2015). Take the P-O/W EMs for an example, when put under an appropriate pH and ionic strength, the proteins around oil droplets can be electrically charged and exert strong electrostatic repulsions to prevent flocculation. At pH values far above or below the pI of the protein, the protein layers of oil droplets are either highly negative or positive charged, which can effectively prevent droplet flocculation via electrostatic repulsion (Li and Tian 2002). Moreover, droplet flocculation may be prevented by steric repulsion as well. For example, a P-O/W EM could

be further stabilized by proper polysaccharides by enhancing the steric repulsion among oil droplets (Kim et al. 2002; Walstra 2002; Dickinson 2003; Guzey and McClements 2006).

Commonly used measurements of flocculation include microscopy methods, particle sizing methods, and methods for detecting physicochemical properties (McClements 2007). For example, optical microscopy is an inexpensive, quick, and informative method to investigate flocculation in emulsions (Mikula 1992). Observations with well-separated and evenly distributed oil droplets in a P-O/W EM would indicate absence of flocculation; otherwise, clumped oil droplets usually indicate the occurrence of flocculation. However, optical microscopy method cannot provide information about flocculation structures. Confocal laser scanning microscopy (CLSM) is more suitable and powerful for that purpose as it exhibits 3D images using appropriate image software tools (Loren et al. 2007). In addition, particle sizing methods such as light scattering can indirectly provide information about flocculation by monitoring the changes in particle size distribution within an emulsion. (McClements 2007). Moreover, changes in physicochemical properties of emulsions (e.g. viscosity and creaming profile) also provide information about flocculation formation (McClements 2015). It is better to combine these methods to obtain a full picture about flocculation.

2.2.2.3 Coalescence

Coalescence happens when two or more oil droplets come in close proximity and merge to form a single larger oil droplet with the partitioning layer disrupted (Tcholakova et al. 2008). During this process, the contact area between oil and aqueous phase is decreased as a result of increased oil droplets size (**Figure 2C**). The rate of coalescence is highly correlated to the interactions between oil droplets, and the physicochemical properties of protein interfacial layers in P-O/W EMs (Walstra 2002; Sanfeld and Steinchen 2008). Controlling coalescence in a P-O/W EMs is generally achieved by preventing oil droplets contact and the rupture of proteins interfacial layer. Oil droplet contact can be reduced by minimizing the duration of contact, ensuring no close contact among oil droplets, and increasing the thickness of the protein interfacial layer (Dickinson 1992; McClements 2015). Rupture of protein interfacial layer can be prevented by lowering the interfacial tension and increasing the thickness and viscosity of the interfacial layer (Dickinson 1992; McClements 2015).

Similar to flocculation, coalescence can be characterized by microscopy and particle sizing methods (Mikula 1992; McClements 2007). In addition, coalescence can be assessed by oiling off test which is based on measuring the amount of oil gathered on the top (McClements 2007). Moreover, accelerated test methods including mechanical agitations

and centrifugations can help to better predict a long-term coalescence stability of an O/W emulsion (McClements 2007). It needs to point out that collecting a representative sample is crucial for these methods especially when the emulsion already starts creaming or is not homogenous, as only a tiny amount of sample is needed.

2.2.3 Oxidative stability of O/W emulsions

Most of the research on emulsion stability has focused on the physical stability because this is directly perceived by consumers. On the other hand, oxidative stability of a P-O/W EM, also referred to as chemical stability, indicates the ability of the emulsion to resist changes in an alteration in the kind of molecules present. Both lipid and protein oxidation are responsible for oxidative instability in a P-O/W EM. The oxidative stability is not only closely connected with the physical stability, but also related to many types of chemical reactions that can induce various adverse outcomes. For example, some lipid oxidation products are surface active that can interact with protein interfacial layer, consequently inducing coalescence of oil droplets and protein oxidation; lipid oxidation can generate off flavor, undesirable color, and potentially toxic oxidative by-products, while protein oxidation might induce quality loss (Lund et al. 2011). Thus, it is indeed necessary to address oxidative stability together with physical stability. The following content focuses on the factors affecting lipid and protein oxidation, and the methods for controlling oxidations in a P-O/W EM.

2.2.3.1 Lipid oxidation

Lipid oxidation involves a complex series of reactions which are usually elucidated as pathways of autoxidation, enzymatic oxidation, and photo-oxidation. In food products, direct photochemical oxidation of lipids is generally of little concern. This is because the light to accelerate lipid oxidation is usually blocked away from lipid by glass or plastic containers (Frankel 2014a). Enzymatic oxidation is not common in processed foods either, because lipoxygenases and other enzymes that catalyze the oxidation of free polyunsaturated fatty acids are mostly deactivated during food processing as a result of thermal degradation (Bartosz 2013). In particular, lipid oxidation in foods is mainly via the autoxidation through free radical chain reactions that generally consists of three steps: initiation, propagation, and termination. Briefly, in the presence of initiators, unsaturated fatty acid (LH) loses a hydrogen atom (H) from an allylic position, thereby forming an alkyl radical (L·) **(Figure 3,** Eq. 1**)**. The initiators can be metal ions, or various oxygen radicals (e.g. alkoxyl radicals (LO·), hydroxyl (HO·), peroxyl radicals (LOO·)). Metal ions either exist naturally in foods or are induced during food processing. Oxygen radicals can be generated either from

oxidation/reduction processes, or from thermal decomposition of hydroperoxides which are contained as impurities in lipid matrices. After the initiation, the alkyl radical quickly reacts with oxygen to form a peroxyl radical, which in turn abstracts a hydrogen atom from another unsaturated fatty acid to form a hydroperoxide as the primary oxidation product and a new alkyl radical for propagations (**Figure 3**, Eq. 2 and 3). Lastly, the autoxidation can be terminated when radicals react between each other to form stable nonradical compounds (**Figure 3**, Eq. 4-7) (Bartosz 2013; Frankel 2014a). The precise mechanism of lipid oxidation in P-O/W EMs may have slight differences compare to bulk oil. Considering hydroperoxides are surface-active compounds, they often accumulate at the interfacial layer around oil droplets. It has been proposed that the decomposition of lipid hydroperoxides at the oil droplet surface into highly reactive radicals is the most likely mechanism for initiating and accelerating lipid oxidation in O/W emulsions (Decker and McClements 2001). Thus, lipid oxidation generally occurs earlier and faster in O/W emulsions than in bulk oil under certain conditions. Particularly in P-O/W EMs, it has been proposed that the protein-structured interfacial region, which is the contact region between oil phase and aqueous phase, plays a critical role in the development of lipid oxidation (Berton-Carabin et al. 2014).

$$LH \rightarrow H + L\cdot \quad \text{(Eq. 1)}$$

$$L\cdot + O_2 \rightarrow LOO\cdot \quad \text{(Eq. 2)}$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot \quad \text{(Eq. 3)}$$

$$LOO\cdot + LOO\cdot \rightarrow LOOR \quad \text{(Eq. 4)}$$

$$L\cdot + LOO\cdot \rightarrow ROOR \quad \text{(Eq. 5)}$$

$$L\cdot + LO\cdot \rightarrow ROR \quad \text{(Eq. 6)}$$

$$L\cdot + H\cdot \rightarrow RH \quad \text{(Eq. 7)}$$

Figure 3. A general scheme for lipid autoxidation (Adapted from Frankel et al. 2005)

Many factors influence lipid oxidation in O/W emulsions, including but not limited to environmental conditions (e.g. temperature, oxygen concentration, pH, salt content, the presence of light, radiations, etc.), fatty acid compositions, oil-phase volume fraction, and interfacial layer properties (Villiere et al. 2005; Kiokias et al. 2006; McClements 2007; Sun and Gunasekaran 2009).

As the aqueous phase accounts for the major part of an O/W emulsion, the composition of the aqueous phase may remarkably influence lipid oxidation (McClements and Decker 2000).

In a simple model of P-O/W EM, the unadsorbed water-soluble proteins play an important role. The unadsorbed proteins can protect lipid phase from oxidation by different mechanisms. Firstly, unadsorbed proteins in the aqueous phase can act as metal chelators or metal binders (Berton-Carabin et al. 2014). They can be used to inhibit lipid oxidation as metal chelators by forming metal complexes, altering the physical or redox state of metal ions, or changing the physical location of metal ions (Pokorny et al. 2001; Elias et al. 2008). Once transition metals are chelated by proteins, initiation of lipid oxidation would be retarded by reducing metal-catalyzed decomposition of lipid hydroperoxides. The chelation ability of a protein depends on environmental pH and its structure. Some proteins remain active within a wide range of pH, while the chelating properties of some proteins are restricted to a specific pH depending on their charges. Secondly, proteins in the aqueous phase can convert hydroperoxides into imines, and sulfur-containing amino acids can reduce hydroperoxides into the inactive hydroxylic derivatives (Pokorny et al. 2001). It was reported that Met even had greater ability than tocopherol to convert hydroperoxides in olive oil (Zalejska-Fiolka 2000). Thirdly, unadsorbed proteins in the aqueous phase can trap or react with free radicals (Wang and Xiong 2005; Kong and Xiong 2006; Sakanaka and Tachibana 2006). It was found that Trp and Cys were preferentially oxidized prior to lipid oxidation in an O/W emulsion system, so that the radical transfer reactions for lipid oxidation was partially inhibited (Elias et al. 2005). Besides, unadsorbed proteins in the aqueous phase can inhibit perceived oxidative rancidity by binding secondary lipid oxidation products and transforming those products into non-volatile compounds. For example, His and Lys are nucleophilic amino acids that can bind to unsaturated aldehydes, and consequently alter the development of rancidity in lipids (Berton et al. 2012).

Apart from the proteins in aqueous phase, proteins at the interfacial layer also play important roles in affecting lipid oxidation. In a P-O/W EM, proteins are the major component for constructing the interfacial layer where lipids and prooxidants such oxygen, transition metals, and free radicals come in close proximity. Thus, the interfacial properties could strongly influence lipid oxidations in the emulsion via different mechanisms (Berton-Carabin et al. 2014). Firstly, proteins at the interfacial layer could affect the oil droplet size. Some results showed that increasing the oil droplet size led to a lower lipid oxidation in P-O/W EMs (Azuma et al. 2009). A larger droplet size corresponds to a smaller interfacial area, which is less favorable for the contacts between oil droplets and prooxidants in the aqueous phase compared to a larger interfacial area. Secondly, proteins at the interfacial layer determine the surface charge of oil droplets. As the transition metals are positive charged, it is assumed

that a positively charged protein-interfacial layer of a P-O/W EM repels the prooxidant metals, thus slowing down the lipid oxidation (Hu et al. 2003). The surface charge is determined by the properties of proteins and the pH of the system. Thirdly, the thickness and packing density of the protein-interfacial layer also impact lipid oxidation. Thicker and denser interfacial layers can efficiently prevent free radicals, oxygen, and other prooxidants in the aqueous phase from reaching the fatty acids in the oil droplets, thereby decreasing lipid oxidation (Gürbüz et al. 2018a). This protective effect mainly depends on the structure of proteins, the packing configuration under certain environmental conditions, and the processing of emulsification. Lastly, proteins at the interface may protect lipid from oxidation as they do in the aqueous phase. The exposure of some antioxidant amino acids as a result of partial denaturation of proteins after loading outside oil droplets may improve the lipid oxidative stability (Berton et al. 2012). The interfacial proteins are preferentially oxidized as a protection of lipids. It needs to point out that neither above mentioned factors is the single determining factor, as lipid oxidation is the result of comprehensive internal and external factors. Under certain conditions, one factor may play a more important role than others. On the other hand, it may be buried by other factors when the condition is changed, even slightly.

There are numerous analytical methods to evaluate lipid oxidation. Generally, they are classified into several types based on what they measure: the absorption of oxygen, the loss of initial substrates, the formation of free radicals, and the formation of primary and secondary oxidation products (Shahidi and Zhong 2005; Frankel 2014b). One parameter to assess the oxidative state of lipid might not be comprehensive. In general, combining several methods with consideration of sample properties, conditions, and experimental purposes provide more reliable results for evaluating lipid oxidation in P-O/W EMs.

2.2.3.2 Protein oxidation

There has been an increasing interest among food scientists in protein oxidation, because protein oxidation is closely associated with the loss of both quality and nutritional value of foods. Protein oxidation will lead to depletion of essential amino acids or to decreasing of their digestibility, which induces loss of nutritional value (Lund et al. 2011). In addition, protein oxidation in P-O/W EMs has often been associated with undesirable changes in protein functionalities such as emulsifying properties. Meanwhile, protein carbonylation caused by oxidation reactions may induce undesirable flavor or odor, as illustrated by Estevez (2011). Moreover, lipid oxidation, which has detrimental effects on food quality is strongly connected with and influenced by proteins. It is noteworthy that protein oxidation

in vivo also contributes to a variety of diseases such as Alzheimer's disease, Parkinson's syndrome, rheumatoid arthritis, muscular dystrophy, cataractogenesis, etc. (Estevez and Luna 2017).

Similar to lipid autoxidation, protein oxidation is usually initiated by reactive oxygen species that originate from irradiation, oxygen, metal-catalyzed systems, peroxides, as well as non-protein radicals and free radicals including hydroxyl radicals ($HO^{\cdot}$), perhydroxyl radicals, and superoxide anion radicals ($O_2^{\cdot -}$). These radicals can effectively initiate formations of protein radicals (Stadtman and Levine 2003). Then, the generated protein radicals are involved in different oxidative processes as follows **(Figure 4)**:

1) Peptide backbone cleavage takes place via two pathways named as α-amidation and diamide pathways in the presence of oxygen. The two pathways yield an amide derivative of the C-terminal amino acid and an α-keto-acyl derivative of the peptide on the N-terminal, and a diamide derivative of the new C-terminal amino acid residue and an isocyanate derivative of the new N-terminal amino acid residue, separately (Stadtman and Levine 2003);

2) Side-chain modifications of amino acid residues result from radicals attacking side-chains of amino acids, and consequently inducing backbone fragmentation, cross-link formation, and generations of unstable derivatives. Several amino acids residues are highly prone to be attacked and oxidized, including Cys and Met with sulfur side-chains, Trp, Tyr, His, and Phe with aromatic side-chains, as well as others such as Lys, Arg, Pro, Leu, Ile, Gly, and Val (Stadtman 1993; Requena et al. 2001);

3) Intra- or inter-protein cross-linking induced oxidative modification of proteins mainly takes place by direct interaction of two carbon-centered radicals, and interaction of two tyrosine radicals. Besides, pathways include oxidation of sulfhydryl groups of Cys, interactions of the carbonyl groups of oxidized proteins with Lys residues, and reactions of both aldehyde groups of malondialdehyde with two different Lys residues yield protein–protein cross linkage as well (Stadtman and Levine 2003).

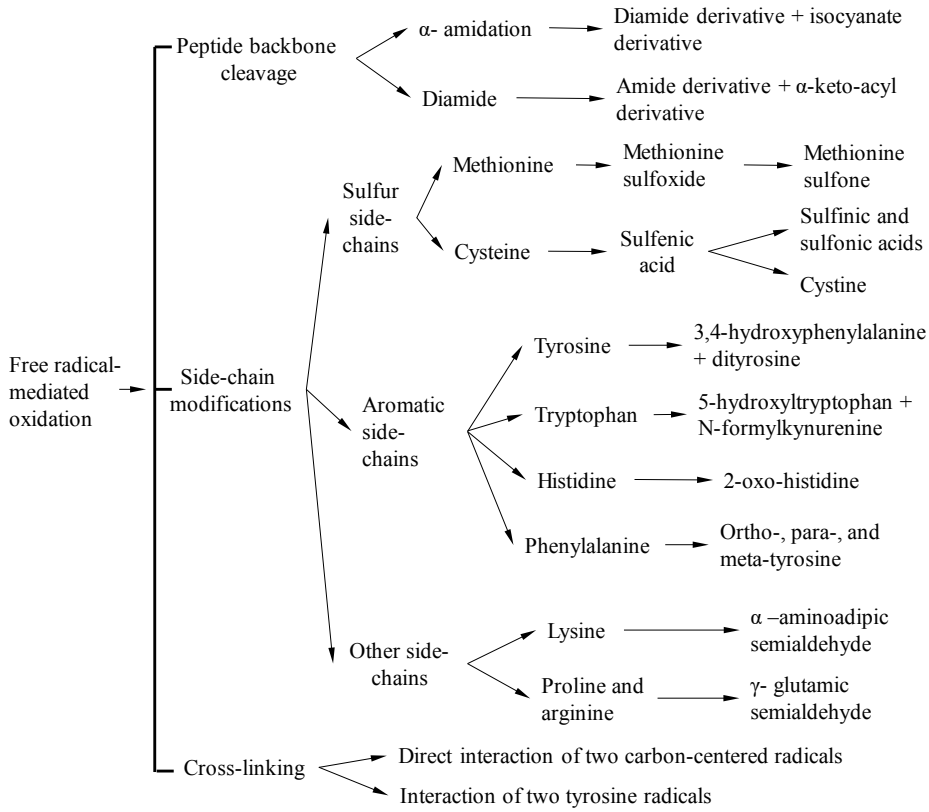

Figure 4. Protein oxidations and their potential oxidative products (Complied from Stadtman 1993, Requena et al. 2001, Stadtman and Levine 2003, Rehder and Borges 2010).

As main ingredients in a simple P-O/W EM, proteins are significantly influenced by lipids within the complex mechanisms of oxidations. Under oxidative conditions, the onset of lipid oxidation normally takes place faster than the oxidation of protein. It is more likely that protein oxidation is initiated and promoted by lipid oxidation products, although other potential pathways cannot be ruled out (Lund et al. 2011). Various protein oxidative products are produced and accumulated after reactions of amino acids/peptides/proteins with lipid-derived oxidative products such as hydroperoxides and aldehydes. Pre-oxidized reactive lipid species serve as sources of radicals and create protein radicals (Lund et al. 2011). For example, protein oxidation is initiated via abstracting hydrogen atoms from protein molecules by peroxyl radicals formed during lipid oxidation. Thus, lipid oxidation plays an important role in protein oxidation.

Measurements of protein oxidation are generally based on monitoring the loss of specific amino acids and the generation of oxidation products. The formation of carbonyl compounds

is one of the most representative results of protein oxidations. The 2,4-dinitrophenylhydrazine (DNPH) method has been widely employed to quantify carbonyl compounds. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) have been utilized to investigate specific carbonyl products from protein oxidation as well. The formation of polymerization via disulfide bridges can be detected visually by SDS-PAGE based on the increase of protein MW. In addition, dityrosine, as a main product of Tyr dimerization, can be sensitively detected by fluorescent spectrophotometer. As thiol groups of Cys are highly susceptible to oxidation, the loss of thiol groups has been adopted as a good indicator for protein oxidation. Determination of thiol groups in proteins is commonly based on the Ellman's reagent (5,5'-dithio-2-nitrobenzoate, DTNB). The method is relatively easy to conduct for water soluble proteins. Trp residues partly lose their native fluorescent characteristics when they are oxidized. Thus, the loss of Trp fluorescence has been employed to monitor protein oxidation as a much simple and sensitive method (Nollet and Toldrá 2008; Armenteros et al. 2009; Lund et al. 2011).

2.3 Protein modifications to enhance stability of P-O/W EMs

Most native plant proteins do not exhibit desired emulsifying properties for food industries, and consequently induce unstable emulsion. Therefore, different modifications of proteins have been studied and developed to improve emulsifying properties (Kilara 1985; Panyam and Kilara 1996). Based on the formation and stabilization of P-O/W EMs, modifications have been focused on altering molecular size, surface charge, hydrophobicity, and molecular flexibility of proteins. Thereby, physical and oxidative stability of emulsions can be improved by enhanced thickness, density, or viscosity of interfacial layers, improved emulsifying capacity of modified proteins, increased protein loading onto the interface, optimized protein conformations, etc. Generally, the modifications of proteins are categorized into physical, chemical, and enzymatic methods.

2.3.1 Physical modifications

Treatments by heating, high-pressure, and altering pH and ionic strength are conventional ways to modify proteins for improving the stability of P-O/W EMs (Tcholakova et al. 2006). Among these treatments, heating is one convenient and easy way. Proper heat treatment of faba bean proteins prior to emulsification can effectively inhibit the activities of native lipoxygenase and peroxidase, thus reduce enzyme-catalyzed lipid oxidation, as illustrated by

Gürbüz et al. (2018b). In addition, appropriate heat treatment can induce conformational changes in proteins, which in turn modifies the surface hydrophobicity of proteins via exposing or burying more hydrophobic amino acid groups. Studies have confirmed that the hydrophobicity of proteins plays critical roles in their emulsifying activity and thereby the stability of emulsions (Karaca et al. 2015). Modulating pH and/or ionic strength can also significantly influence fractionation, solubility, and emulsifying properties of proteins (Mitidieri and Wagner 2002). Moreover, pH may alter chelating-ability of proteins, which could either increase or decrease the oxidative stability of emulsions via controlling the distribution of transition metals.

Recent studies have shown that connecting polysaccharides with proteins via electrostatic interaction is another important and effective way of physical modifications. Polysaccharides can be added into a protein solution or a protein-emulsified emulsion. In general, protein-polysaccharide complexes could be formed first via electrostatic interaction and then introduced as a new emulsifier (as complex model); or, the polysaccharides could be added to existing protein-emulsified O/W emulsions to form an extra outer layer around the droplets (as layer-by-layer (LBL) model) (Berton-Carabin et al. 2014). The two different preparation methods (complex *vs.* LBL model) are expected to have a major impact on the interfacial properties, especially the thickness and compactness (Berton-Carabin et al. 2014).

A number of studies have shown that polysaccharides could help protein-emulsifiers to form a thicker interfacial layer around oil droplets (Wooster and Augustin 2006; Jourdain et al. 2009; Wong et al. 2011), thereby establishing a stable emulsion against droplet aggregation via protein and polysaccharide interaction (Berton-Carabin et al. 2014). For example, adding xanthan gum to wheat protein-emulsified emulsions improved emulsion stability to high ionic strengths (Qiu et al. 2015). Corn fiber gum-bovine serum albumin (CFG-BSA) conjugate-emulsified emulsion showed better stability in comparison to single BSA or CFG emulsion, attributing to its higher amount of adsorbed proteins and polysaccharides (Liu et al. 2018). Emulsions emulsified with soluble glycinin-chitosan complex displayed improved stability at certain ratio due to synergistic effect of the two molecules (Yuan et al. 2014). The combination of Na-caseinate and alginates under certain ratio allowed the production of oxidatively stable emulsions (Yesiltas et al. 2017). Conversely, some studies showed that additions of cationic polyelectrolytes to protein-emulsified emulsions promoted droplet aggregation, creaming instability, and viscosity enhancement. The opposite conclusions might be mainly attributed to the different experiment conditions such as the type and the ratio of polysaccharides and proteins, environmental pH and ionic strength, and preparing

methods, etc. Some published studies on plant protein-polysaccharide combination to stabilize emulsions are listed in **Table 2**.

Table 2. Recent published studies on plant protein-polysaccharide combination to stabilize O/W emulsions.

Protein	Polysaccharide	Reference
Glycinin	Chitosan	Yuan et al. 2014
Maize protein	Xanthan gum	Makri and Doxastakis 2006
Potato protein	Chitosan	Calero et al. 2013
Lupin protein	Chitosan and xanthan gum	Burgos-Disa et al. 2016
Soy protein	Rutin	Cui et al. 2014
Soy protein	Alginate and chitosan	Zhang et al. 2015
Soy protein	Soy polysaccharide	Yin et al. 2012
Soy and flaxseed protein	Gum arabic	Wang et al. 2011
Pea protein	Pectin	Aberkane et al. 2014
Pea protein	Chitosan	Elmer et al. 2011
Pea protein	Pectin and chitosan	Gharsallaoui et al. 2010
Pea protein	Gum arabic	Liu et al. 2010
Whey protein	Chitosan	Moschakis et al. 2010
Wheat protein	Pectin and xanthan gum	Qiu et al. 2015
Wheat protein	Dextran	Wong et al. 2011
Whey protein	Flaxseed gums	Khalloufi et al. 2009
Whey protein	Chitosan	Laplante et al. 2006
Whey protein	Gum arabic	Klein et al. 2010

2.3.2 Chemical modifications

Chemical modifications are effective and efficient methods to optimize emulsifying properties of proteins. However, some chemical modifications should be considered with caution, or even refrained from food related products due to safety concerns. Chemical modifications usually include alkylations (e.g. Lys, Cys, Met, and His), oxidations (e.g. Cys, Met, His, and Trp), acylations (e.g. Lys and Tyr), esterifications, and amide formations (e.g. Gln and Asn), which affect the conformation, hydrophobicity, charge distribution, and net charge of proteins (Lundblad 2014). Many studies reported that chemical modifications could improve one or several properties of either proteins or emulsions such as the solubility of proteins, the rheological behavior of emulsions, foaming capacity of emulsions, etc. (Beuchat 1977; Woo and Richardson 1983; Oliver et al. 2006). Maillard reaction is the one of mostly used chemical modifications for improving some specific properties of emulsions. It is achieved by the reaction between amino groups from proteins and carbonyl groups from

polysaccharides. Covalent attachment of polysaccharides to proteins can increase steric layer thickness, and consequently improve stability of emulsions against calcium induced flocculation (Wooster and Augustin 2006).

2.3.3 Enzymatic modifications

Enzymatic modifications of proteins are favored by commercial manufacturers because of food safety, lower costs, easier control of the reactions, and acceptability by consumers and regulatory agencies (Schwenke 1997). Enzymatic modifications of proteins have been shown to improve the emulsifying properties and the stability of corresponding emulsions. In general, enzymatic modifications of proteins applied in P-O/W EMs are either via decreasing the molar mass of proteins by proteolytic enzymes, or increasing the molar mass by cross-linking enzymes (Chen et al. 2011c; Luisa et al. 2015)

Proteolytic enzymes can cleave the peptide linkage of proteins at specific or non-specific points, and yield two peptides (Tavano 2013). Enzyme specificity, protein confirmation, enzyme dose, and environmental conditions (e.g. pH, ionic strength, temperature, other components such as inhibitory substances, etc.) all together influence the hydrolyzing process. Hydrolysis could increase solubility of proteins by producing small peptides, and correspondingly increasing the ionizable amino and carboxyl groups. Chen et al. (2011c) suggested that increased protein solubility and decreased MW were the main reason for the greatly improved emulsifying capability of soy protein isolate hydrolysates. However, the hydrolysis process needs to be well controlled as excessive hydrolysis may accelerate the desorption of proteins/peptides at interfacial layer, and thereby decrease stability of emulsions (Schroder et al. 2017). In addition, hydrolysis could modify protein hydrophobicity which has a major impact on the emulsifying properties of proteins (Wu et al. 1998). Due to the amphiphilic properties, proteins can self-aggregate, and form continuous and homogeneous membranes around oil droplets. Suitable hydrolysis could increase surface hydrophobicity and expose more buried hydrophobic groups, thus improving the adsorption of proteins at the interface and emulsifying capability. It is noteworthy that excessive hydrolysis may cause a drastic decrease in hydrophobicity due to cleavage of hydrophobic bonds resided at the surface. As a result, the emulsifying capacity of proteins would be impaired (Tavano 2013). Besides, protein hydrolysates exhibit higher capacity to inhibit lipid oxidation, indicated by higher metal-chelating activities and radical-scavenging activity (Pokorny et al. 2001; Davalos et al. 2004) . Thus, hydrolyzed proteins may be utilized in P-O/W EMs to retard lipid oxidation. In particular, Alcalase-derived hydrolysates show higher antioxidant activities and are more resistant to digestive enzymes

than those from other peptidases (Sarmadi and Ismail 2010). Furthermore, protein hydrolysates show better hypoallergenic and high-tolerance properties than native proteins (Mahmoud et al. 1992). However, it should be stressed that both insufficient and extensive hydrolysis might impair functionalities of proteins. It is critical to determine the optimum degree of hydrolysis (DH) so that a best stable P-O/W EM can be established. Choosing optimal reaction conditions such as pH and temperature based on the type of enzyme is always the prerequisite. **Table 3** lists a wide variety of commonly used hydrolysis enzymes. They can produce peptides with various MWs or unhydrolyzed proteins via different cutting points (Tavano 2013).

Table 3. Commonly used hydrolysis enzymes and their preferential cleavages.

Protease	Hydrolysis reaction
Aminopeptidase A	Release of N-terminal Leu and Pro, but not Arg or Lys.
Aminopeptidase B	Release of N-termianl Arg and Lys from oligopeptides when P1' is not protein.
Carboxypeptidase A	Release of a C-terminal amino acid, but little or no action with -Asp, -Glu, -Arg, -Lys or -Pro.
Carboxypeptidase B	Preferential release of a C-terminal Lys or Arg.
Chymotrypsin	Preference release of N-terminal Tyr, Trp, Phe, Leu at P1 position.
Papain	Preference for an amino acid bearing a large hydrophobic side chain at the P2 position.
Pepsin	Preferential cleavage: hydrophobic, preferably aromatic residues.
Subtilisin	Hydrolysis of proteins with broad specificity for peptide bonds, and a preference for a large uncharged residue in P1.
Thermolysin	Preferential release of C-terminal Leu and Phe at P1 position.
Trypsin	Preferential release of N-terminal Arg and Lys at P1 position.

Adapted from Tavano 2013.

On the other hand, cross-linking enzymes can catalyze inter- or intramolecular cross-linking reactions of proteins. Common enzymes used for cross-linking are microbial transglutaminase (MTG), laccase/peroxidase and lysyl oxidase (Færgemand et al. 1998). For example, MTG catalyzes acyl transfer reactions between γ-carboxyamide of peptide or protein-bound glutamyl residue (acyl donor) and primary amino group (acyl acceptor). When Lys residues act as acyl acceptor, ε-(γ-glutamyl)- lysine "isopeptide" covalent bonds are formed, resulting in polymerization or amine incorporation (Luisa et al. 2015). It was shown that the surface shear viscosity and the dilatational elasticity of caseinate or β-lactoglobulin were increased after cross-linking by MTG (Faergemand and Murray 1998). As a result of

suitable cross-linking of proteins, texturization, foaming, whipping, emulsifying properties, and oxidative stability of P-O/W EMs can be improved. In FBPI-emulsified O/W emulsions, limited cross-linking by MTG led to a higher physical stability towards coalescence or flocculation, as a result of increased net surface charge (Schwenke 2001b). Besides, the stability of emulsions toward lipid oxidation was improved as well, which may be attributed to thicker interfacial layer around oil droplets, larger emulsion droplet size, and protective effect of proteins (Azuma et al. 2009; Berton-Carabin et al. 2014). On the other hand, cross-linking of proteins may induce opposite results. Yildrim et al. (1996) showed that the emulsifying property of cross-linked whey protein isolates was lower than that of native one as a result of multi-factors including hydrophobicity, electrostatic, hydration repulsions, and steric interactions in emulsions. Kellerby et al. (2006) reported that emulsions with more cohesive MTG cross-linked casein at the interface of oil-water did not show better lipid oxidative stability compared to untreated emulsions. This might be attributed to the ability of prooxidants in the aqueous phase to diffuse through the interfacial layer where they may have longer time to contact with lipid hydroperoxides and promote the decomposition into free radicals. Consequently, more free radicals would induce lipid oxidation in the droplet core. Similar to hydrolysis, it should be emphasized that choosing an optimum level of enzymatic treatment for proteins is important for achieving desired functionalities. Either insufficient or extensive cross-linking may result in unexpected outcomes.

3 Aims of the study

The overall aim of the research was to study how different modifications of FBPI affect the physical and oxidative stability of O/W emulsions.

The specific objectives were:

1. To investigate the effects of MTG treatment on physiochemical properties and emulsifying functionality of FBPI (study I)

2. To study the effects of enzymatic hydrolysis of FBPI by Alcalase on the physical and oxidative stability of O/W emulsions (study II)

3. To investigate how the assembling of the interfacial layer affects the physical and oxidative stability of FBPI-emulsified O/W emulsions with chitosan (CH) (study III)

4 Materials and methods

4.1 Materials

Faba beans cultivar "Divine" 2012 (for study I and II) and "Kontu" 2016 (for study III) were grown at Viikki Experimental Farm of the University of Helsinki in Finland. Rapeseed oil (Keiju Rypsiöljy, Bunge Finland Ltd., Raisio, Finland) was purchased from a local store. MTG (ACTIVA®-WM, with 99% maltodextrin with, ~ 80 U/g activity) in study I was kindly provided by Ajinomoto Food Europe S.A.S. (Mesnil-Saint-Nicaise, France). Alcalase (2.4 L FG, 2.22 AU/g activity) in study II was provided by UNIVAR (Vantaa, Finland). Chitosan (CH, MW between 190-310 kDa, degree of deacetylation: 75-85%) in study III was purchased from Sigma-Aldrich (Steinheim, Germany).

Aluminum oxide (Al_2O_3, 90 active neutral, activity stage I, for column chromatography, 0.063-0.200 mm, 70-230 mesh ASTM) was acquired from Merck KGaA (Darmstadt, Germany). Sodium dodecyl sulfide polyacrylamide gels (NuPAGE 12% Bis-Tris) for study II were purchased from Invitrogen (CA, USA). All other chemicals were obtained from either Sigma-Aldrich (Steinheim, Germany) or Merck (Darmstadt, Germany), including bovine serum albumin (BSA), linoleic acid (≥99%), tocopherol standards (α-, β-, γ-, δ-tocopherol), sodium hydroxide (NaOH), hydrochloric acid (HCl), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), guanidine hydrochloride (CH_6ClN_3), sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA) and sodium azide (NaN_3), etc.

4.2 Extraction of FBPI

Fine faba bean flours with an even particle size were obtained by milling dehulled faba beans with a high speed rotor ultra-centrifugal mill (sieve pore size of 0.5 mm, Ultra Centrifugal Mill ZM 200, Retsch, Germany). FBPI was extracted by repeated isoelectric precipitation (Damodaran and Kinsella 1981). Briefly, faba bean flour was dissolved in Milli-Q water (flour: water =1:10, w/v) adjusted to pH 8.0 with 2 N NaOH, and stirred at room temperature for 4 hours. After centrifugation, the supernatant was adjusted to pH 4.5 with 2 N HCl. The pellet collected after centrifugation was re-dissolved in water and re-adjusted to pH 8.0 overnight. The acid precipitation and alkaline dissolution were repeated for three times. Afterwards, the pellet collected was dialyzed against water at pH 8.0 for 48 hours using a

molecular porous membrane tubing (Spectra/Por® MWCO 6-8000, Spectrum Medical Devices, Rancho Dominguez, CA, USA), and then lyophilized on a freeze-dryer (FTS Systems Inc., Stone Ridgeny, NY, USA). The yield of FBPI was determined by the Biuret method. FBPI was stored at -20 °C for further use.

4.3 FBPI modifications

4.3.1 MTG treatment (study I)

MTG-treated FBPI (MTG-FBPI) was prepared as previously described with minor modifications (Damodaran and Agyare 2013). FBPI dispersions in Milli-Q water were incubated with MTG (5 U/g of protein substrate) at 37 °C with constant stirring for 60 min (MTG60-FBPI), 120 min (MTG120-FBPI), or 240 min (MTG240-FBPI). A control-FBPI named as MTG0-FBPI was prepared by adding NH_4Cl-inactivated MTG (addition of NH_4Cl to a final concentration of 10 mM) to FBPI dispersions. FBPI dispersion without MTG was used as native-FBPI.

4.3.2 Alcalase treatment (study II)

FBPI was hydrolyzed by Alcalase to DHs of 4% (DH4-FBPI), 9% (DH9-FBPI), and 15% (DH15-FBPI) as previously described with minor modifications (Ghribi et al. 2015). Briefly, Alcalase (0.01 AU/g protein) was added to 5% (w/v) FBPI dispersions pre-equilibrated at pH 8.0 and 50 °C. Then, the reaction was maintained at 50 °C and pH 8.0 by the continuous addition of 0.2 M NaOH. The total added volume of NaOH to reach each target DH was calculated based on the equation: $DH(\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times N_b}{MP} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$, where the DH is the percent ratio of the number of peptide bonds cleaved (h) to the total number of peptide bonds in the protein substrate (h_{tot}, assumed to be 7.8 meqv/g protein for faba bean protein (Adlernissen et al. 1983)); B is the base consumption (mL); N_b is the normality of the base; MP is the mass of the protein (g); α is the average degree of dissociation of the α-NH_2 amino groups released during the hydrolysis which is assumed to be 0.885 at pH 8.0 and 50 °C (Ghribi et al. 2015). After target DHs were reached, hydrolysis was stopped by heating immediately at 80 °C for 20 min to inactivate the enzyme (Ghribi et al. 2015). Finally, the supernatant containing hydrolysates was recovered by centrifugation at 5000 × g for 20 min at 4°C. A FBPI dispersion treated with heat-inactivated Alcalase served as a control (DH0-FBPI). A FBPI dispersion without Alcalase served as a native-FBPI control.

4.3.3 Combination with CH (study III)

To prepare the soluble FBPI-CH complex, a FBPI:CH ratio of 10:1 was adopted to avoid bridging or depletion-induced flocculation due to insufficient or excessive CH (Burgos-Diaz et al. 2016). Firstly, solutions of FBPI (1%, w/v) and CH (0.1%, w/v) in sodium acetate buffer (100 mM, pH 3.0) were prepared separately, and then mixed at 1:1 (v/v). At pH 3.0, both FBPI and CH carried positive charges and did not interact electrostatically (Klinkesorn 2013). After that, with continuous addition of NaOH to the mixture, FBPI and CH started to pose opposite charges, interacted electrostatically, and produced soluble complexes and insoluble coacervates successively. To determine the critical pH values for soluble FBPI-CH complexes (pH_c) and insoluble coacervates ($pH_{\phi 1}$), pH was plotted against the turbidity at 600 nm during the NaOH-titration (Elmer et al. 2011). A pH range between 4.5-5.0 for forming soluble FBPI-CH complexes was determined graphically based on the titration curve (**Figure 5**). A pH of 4.8 was selected to prepare the soluble FBPI-CH complex dispersion in the following experiments.

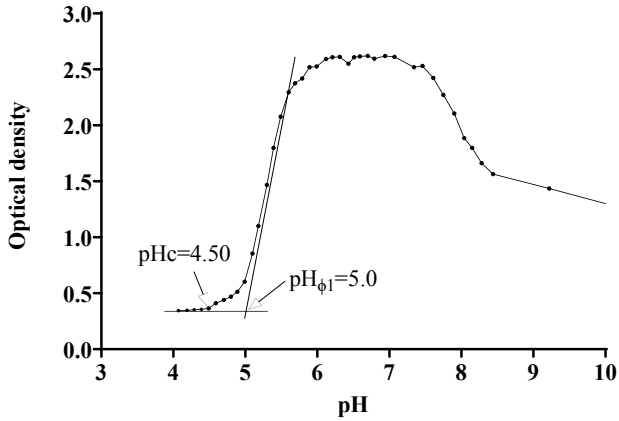

Figure 5. Titration curve as a function of pH for a 10:1 FBPI-CH mixture, showing the graphical interpretation of pH-induced structure forming events associated with the formation of soluble FBPI-CH complexes (pH_c) and insoluble coacervates ($pH_{\phi 1}$).

4.4 Physiochemical properties of modified FBPI

4.4.1 Electrophoresis (studies I-II)

SDS-PAGE under reducing conditions was employed to examine MW changes of FBPI after enzymatic modifications (Agyare and Damodaran 2010). A 4-20% gradient acrylamide separating gel and a 4% acrylamide stacking gel were used for MTG treated FBPI (study I), and a 12% SDS-PAGE (NuPAGE® Bis-Tris, Invitrogen) was used for Alcalase treated FBPI

(study II). Protein samples for SDS-PAGE were prepared by mixing 0.2% (w/v) of native FBPI, control-FBPI, and enzymatic-treated FBPI with an equal-volume of sample buffer, and then heated in boiling water for 3 min. Then, prepared protein samples and pre-stained protein marker were loaded into each lane of the gel. The electrophoresis run on a Mini-PROTEAN 3 apparatus (Bio-Rad laboratories, Hercules, CA, USA) at 200 V for 60 min. Protein bands were stained using Coomassie brilliant blue. The MWs of unknown proteins were estimated via regression between the logs of standard MWs and the relative mobility of the protein markers.

4.4.2 Surface charge (Zeta potential) (studies I-III)

The overall surface charges of control-FBPI, enzymatic-treated FBPI and soluble FBPI-CH complex were determined by measuring the electrophoretic mobility (U_E) of protein dispersions (0.2%, w/v) at desired pH using a Zetasizer Nano-ZS90 instrument (Malvern Instruments, Westborough, MA). U_E was used to calculate the zeta potential as follows:

$$U_E = \frac{2\varepsilon * \xi * f(\kappa\alpha)}{3\eta}$$

where ε is the permittivity, $f_{(\kappa\alpha)}$ is a function related to the ratio of particle radius (α) and the Debye length (κ), and η is the dispersion viscosity. The Smoluchowski approximation $f_{(\kappa\alpha)}$ equaled to 1.5.

4.4.3 Surface hydrophobicity (studies I-III)

Surface hydrophobicity (S_0-ANS) of control-FBPI, enzymatic-treated FBPI and soluble FBPI-CH complex were measured at desired pH as previously described with minor modifications (Karaca et al. 2011). Sample dispersions were diluted with 10 mM sodium phosphate buffer (SPB, pH 7 or 8) to give five gradient concentrations ranged from 0.005% to 0.025% (w/v). To 3.2 ml of these solutions, 32 µl of the fluorescent probe, 8-anilino-1-naphthalenesulfonic acid (ANS: 8 mM ANS solution in 10 mM SPB) was added, followed by vortexing for 5 s. Samples were kept in the dark for 5 min. Fluorescence intensity (FI) was then measured by a LS 55 Luminescence Spectrometer (PerkinElmer Inc., Waltham, MA, USA) with excitation and emission wavelengths at 390 and 470 nm, respectively. The slit width was set to 2.5 nm. The FI attributed to protein in the buffer was subtracted from the FI of each sample with ANS. The initial slope of the FI versus protein concentration was calculated by linear regression analysis to represent protein surface hydrophobicity.

4.4.4 Protein solubility (studies I-II)

Control and enzymatic-treated FBPI dispersions (1%, w/v) (pH 7.0 in study I and pH 8.0 in study II) were stirred magnetically for 10 min. Then the dispersions were centrifuged at 12000 × g for 20 min at 20 °C. After appropriate dilutions, the protein content of the supernatant was determined by the Biuret method. The protein solubility was expressed as percentage of supernatant protein over total protein.

4.4.5 Interfacial tension (study III)

The abilities of surface-active FBPI (pH 3.0) solution and soluble FBPI-CH complex (pH 4.8) solution to lower the interfacial tension were determined against purified rapeseed oil by a du Nouy tensiometer (KSV Sigma 70, KSV, Finland) with a platinum ring. Briefly, 15 mL of the solution (heavy phase) and 15 mL of oil (light phase) were loaded sequentially into a glass well. The two phases were allowed to equilibrate for 20 min for avoiding any shaking. For the measurement, the ring moved from the solution to oil at the speed of 2 mm/min. Based on the maximum force to separate the ring from the interface, the interfacial tension of solutions was calculated and recorded. In addition, FBPI and CH mixture at pH 3.0 were measured.

4.5 Preparations of emulsions

4.5.1 Emulsions prepared with enzymatic-treated FBPI (studies I-II)

Emulsions of MTG0 (control), MTG60, MTG120, and MTG240 (study I) were prepared by MTG0-FBPI, MTG60-FBPI, MTG120-FBPI, and MTG240-FBPI, respectively. Emulsions of DH0, DH4, DH9, and DH15 (study II) were prepared using DH0-FBPI, DH4-FBPI, DHFBPI and DH15-FBPI, respectively. The MTG-related emulsions consisted of 3% (w/v) of proteins and 10% (w/v) purified rapeseed oil, while Alcalase-related emulsions were made of 1% and 5%, respectively. Briefly, a coarse emulsion was prepared by blending different FBPI dispersions and purified rapeseed oil for 2 min using an Ultra-Turrax® T25 homogenizer at 13500 rpm (IKA®-Werke GmbH & Co. KG, Staufen im Breisgau, Germany). Purified rapeseed oil was obtained by chromatographic purification as previously described to eliminate any detectable residual tocopherols (verified by normal-phase HPLC with a fluorescence detector) (Lampi et al. 1999). Then, a more stable emulsion was obtained by homogenizing the coarse emulsion by an M-110Y Microfluidizer® processor (Microfluidics™, MFIC Corp., Westwood, MA, USA) at an operating pressure of 600 bars for 10 min. Next, NaN_3 was added to a final concentration of 0.02% (w/v) to inhibit microbial

growth. Finally, the emulsion was equally divided into three sealed vials and stored at 37 °C in dark with constant magnetic stirring. Samples were collected on day 0, 1, 4, and 7 to determine the physical and oxidative stabilities.

4.5.2 Emulsions by combination of FBPI and CH (study III)

In order to assess how different assembly of FBPI and CH affects the interfacial properties and emulsion stability, emulsions namely EMN (emulsified by FBPI, and non-interactive CH in aqueous phase), EML (first emulsified by FBPI, then stabilized by a second layer of CH via electrostatic attraction, as LBL model), and EMC (emulsified by the soluble FBPI-CH complex, as complex model) were prepared. EMFB was emulsified by FBPI at pH 3.0, serving as a control (**Figure 6**).

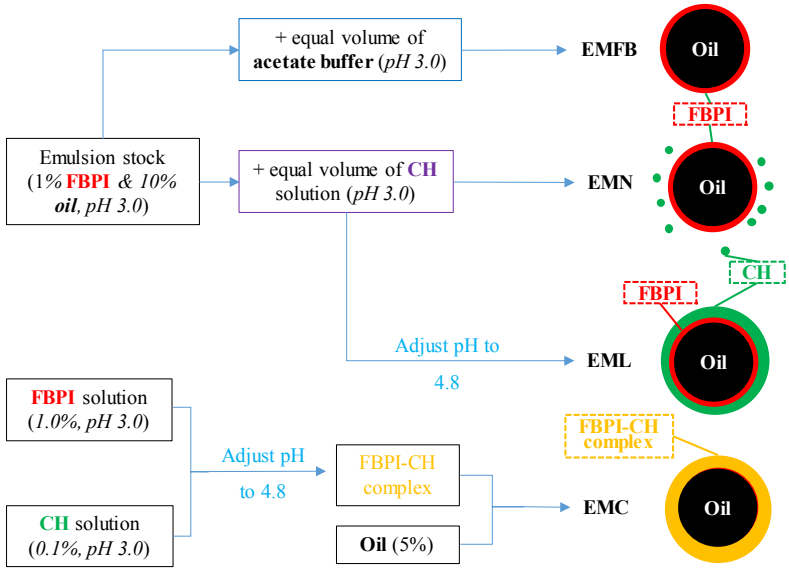

Figure 6. The formulation of O/W emulsions by FBPI and CH. EMFB (emulsified by FBPI at pH 3.0), EMN (emulsified by FBPI, with the presence of free CH in aqueous phase at pH 3.0), EML (first emulsified by FBPI, then stabilized by a second layer of CH via electrostatic attraction at pH 4.8), and EMC (emulsified by soluble FBPI-CH complex at pH 4.8).

To be more specific, to prepare EMC, soluble FBPI-CH complex (0.5-0.05%, w/v, pH 4.8) and purified rapeseed oil (5%, w/v) were homogenized as described in **chapter 4.5.1** except for using 200 bars instead of 600 bars. To prepare EMFB, EMN, and EML, a stock emulsion with FBPI (1%, w/v, pH 3) and purified rapeseed oil (10%, w/v) was first made as described for EMC. Then, the stock emulsion was diluted with either equal volume of acetate buffer (pH 3.0) or CH solution (0.1%, w/v, pH 3.0) to prepare EMFB and EMN. To formulate EML, the pH of the above made EMN was adjusted to 4.8 by adding NaOH, so that CH was

bonded to the adsorbed FBPI on oil droplet via electrostatic attraction. The freshly made emulsions were stirred for 30 min for equilibration at room temperature. Then, emulsions samples were divided and ready for measurements as described in **chapter 4.5.1**.

4.6 Interfacial layer properties

4.6.1 Protein adsorption fraction (F_{ads}) (studies II-III) and surface load (Γ_s) (study II)

Unadsorbed and adsorbed proteins in study II and III were recovered as previously described with minor modifications (Yang and Xiong 2015). Briefly, 5 mL emulsion was centrifuged at 35000 × g for 60 min at 4 °C. The bottom aqueous phase and precipitates containing unadsorbed proteins were carefully collected for measurement of protein content by the Biuret method. Protein adsorption fraction (F_{ads}) refers to the fraction of protein adsorbed onto the droplets and is calculated as follows (Chen et al. 2011b): $F_{ads} = \frac{C_i - C_{aq}}{C_i} \times 100\%$, where C_i is the initial protein concentration per unit volume of emulsion (kg/m^3), and C_{aq} is the unadsorbed protein concentration per unit volume of emulsion (kg/m^3). The surface load (Γ_s) of emulsions with Alcalase-treated FBPI (study II) was calculated as follows (McClements 2007): $\Gamma_s = \frac{(C_i - C_{aq}) \times d_{3,2}}{6\Phi}$, where C_i and C_{aq} are the same as those in equation for F_{ads}; $d_{3,2}$ is the surface mean diameter determined as described in **chapter 4.7.2**; Φ is the oil volume fraction (0.05).

4.6.2 Interfacial layer thickness (study III)

Polystyrene latex beads were used to determine the interfacial layer thickness of emulsions in study III according to Wong et al. (2011). The beads were charge-stabilized with a zeta-potential -52.6 ± 1.8 mV and a diameter of 112.7 ± 0.4 nm. The latex bead was diluted to 0.05 wt% with acetate buffer (pH 3.0). FBPI, CH and soluble FBPI-CH complex solutions were prepared as described in **chapter 4.3.3**. Further dilution was conducted to achieve the desired concentration for FBPI (0.0025-0.1 mg/mL) and CH (0.00025-0.01 mg/mL). To mimic the interfacial layer of EMFB, EMN, and EMC, 3.9 mL of the diluted FBPI solution, the diluted FBPI & CH solution (1:1, v/v), or the diluted soluble FBPI-CH complex was mixed with 300 μL of latex bead suspension. To mimic interfacial layer of EML, 1.95 mL of the diluted FBPI solution was firstly mixed with 300 μL of latex bead suspension. After equilibration for 10 min, 1.95 mL of the diluted CH solution was added. Then, the mixture was adjusted to pH 4.8 to promote the second layer formation by binding CH to the adsorbed FBPI. The size was measured using a Zetasizer Nano-ZS90 instrument (Malvern Instruments,

Westborough, MA) after 2h equilibration at room temperature. All the measurements were performed using a 633 nm laser at a scattering angle of 173°. The intensity z-average size was recorded. The interfacial layer thickness was calculated as the differences in latex bead diameter with or without adsorbed layers.

4.6.3 Visualization of CH and FBPI (study III)

The distribution of CH and FBPI at the interface and in the aqueous phase of fresh emulsion in study III was visualized by a Leica TCS SP5 confocal laser scanning microscope (CLSM) (Leica Microsystems, Heidelberg, Germany). To get better visualization of both FBPI and CH simultaneously, fluorescein isothiocyanate (FITC)-labeled CH was first synthesized, as descried by Qaqish and Amiji (1999) with minor modifications. In brief, 50 mL of 2.0 mg/mL of FITC in methanol and 100 mL dehydrated methanol were added into 100 mL of CH in acetate solution (1% w/v). After 3h of reaction at room temperature in the dark, the pH of the mixture was adjusted to 10 to precipitate the FITC-CH complex. To remove unreacted FITC, the precipitate was washed with Milli-Q water and centrifuged until no fluorescence signal was detected in the supernatant. Then, the FITC-CH was dissolved in acetate buffer, dialyzed against water for 4 days, and freeze-dried. Emulsions were prepared as described in **chapter 4.5.2**, with FITC-CH as a substitute for CH. For protein staining, 5 ml of freshly made emulsion was mixed thoroughly with 20 μL of Fast Green FCF (1mg per mL of Milli-Q water) (Gallier et al. 2010). Two drops of emulsions were placed on a 35 mm glass-bottom dish (Nunc) and observed under a 63 × oil objective. The laser excitation wavelengths were set at 633 nm and 488 nm for Fast Green FCF and FITC, respectively.

4.6.4 Interfacial low-shear viscosity (study III)

The shear rate dependency of the steady-state viscosity of emulsions in study III were performed under shear rates range of 0.03 to 100 1/s by a HAAKE RheoStress rheometer (RS 50, HAAKE Rheometer, Karlsruhe, Germany) at 25 ˚C. The cone and plate geometry with 35 mm diameter and 2° cone angle were used. The low-shear viscosity was obtained from reading the viscosity at a fixed low-shear rate of 0.03 1/s, where the maximal viscosity was observed (Laplante et al. 2006).

4.7 Physical stability of emulsions

4.7.1 Emulsifying activity index (EAI) (study I)

A 100-µl emulsion sample from study I was immediately (0 min) removed from the bottom of the homogenized emulsion and added to 7.5 mL of 10 mM SPB (pH 7.0) containing 0.1% SDS. After vortexing for 5 s, the absorbance of the diluted emulsion was measured at 500 nm using a UV/Vis spectrophotometer (PerkinElmer Inc., Waltham, MA, USA). EAI was calculated as follows:

$$EAI\ (^{m^2}/_{g}) = \frac{2 * 2.303 * A_0 * DF}{C * \emptyset * \theta * 10{,}000}$$

where DF is the dilution factor, C is the initial concentration of protein (g/mL), Ø is the optical path, θ is the fraction of oil used to form the emulsion, and A_0 is the absorbance of the diluted emulsions at 0 min.

4.7.2 Droplet size (studies I-III)

The size distribution of emulsion droplets was determined at room temperature after appropriate dilution on a laser light scattering instrument (Mastersizer 3000, Malvern Mastersizer, Malvern Instruments, Ltd., Worcestershire, UK) with a hydro EV dispersion unit. The surface mean diameter ($d_{3,2}$) of oil droplets was recorded in all studies, and the volume mean diameter ($d_{4,3}$) was also recorded in study III.

4.7.3 Morphology (studies I-III)

A drop of emulsion was placed on a slide glass and covered with a cover glass. The microstructure of emulsions was observed at room temperature using an optical microscope (Axio Scope A1, Carl Zeiss Inc., Oberkochen, Germany) equipped with an AxioCam camera under 100 × objective.

4.7.4 Backscattering (BS) and Turbiscan stability index (TSI)

The physical stability of emulsion in study II and III was also monitored by a Turbiscan Lab Expert analyzer (Formulaction, France) for 7 days at 25 °C. The vials containing 20 mL of emulsions were scanned from the bottom to the top by a light beam emitted in near infrared light (λ = 880 nm). Detectors that moved synchronously along the sample height measured the intensity of transmitted and back scattered light at 180° and 45°, respectively. The analysis of stability was carried out as a variation of delta backscattering (ΔBS) calculated as the difference between backscattering intensity at 0 h and a given time. Turbiscan stability index (TSI) was calculated with Turbiscan software version 1.2 and an increase in TSI

indicated decreased system stability (Santos et al. 2016). TSI is the sum of all the scan differences in the measuring cell calculated as follows: $TSI = \sum_j \left| scan_{ref}(h_j) - scan_i(h_j) \right|$, where $scan_{ref}$ and $scan_i$ are the initial backscattering value and the backscattering value at a given time, respectively, h_j is a given height in the measuring cell (Santos et al. 2016).

4.8 Lipid oxidation (studies I-III)

4.8.1 Conjugated dienes (CDs)

CDs was measured as previously described with minor modifications (Estevez et al. 2008). Briefly, 100 µL of emulsion was dissolved with 1.5 mL of isooctane/isopropanol (2:1, v/v) and vortexed for 30 s vigorously. After centrifugation at 550 × g for 5 min at 4 °C, 200 µL of upper organic phase was collected, diluted, and vortexed with 4.8 mL isooctane. Then, the absorbance was measured at 234 nm by a UV/Vis spectrophotometer (PerkinElmer Inc., Waltham, MA, USA). The CDs concentration was calculated using 25200 $M^{-1}cm^{-1}$ as the molar extinction coefficient.

4.8.2 Secondary volatile by-products

The volatile oxidation products were measured by headspace solid-phase micro extraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) as described (Damerau et al. 2014). The system comprised of an HS-SPME injector (combiPAL, CTC Analytics, Lake Elmo, MN, USA) with a DVB/CAR/PDMS-fiber (50/30 µm film thickness; Supelco, Bellefonte, PA, USA), GC (HP 6890 series, Agilent Technologies Inc., Wilmington, DE, USA) equipped with a capillary column SPB-624 (30 m × 0.25 mm i.d., 1.4 µm film thickness; Supelco, Bellefonte, PA, USA) and MS detector (Agilent 5973 Network, Agilent Technologies Inc., Wilmington, DE, USA). Peak areas were recorded. Identification of the volatile compounds was based on matching mass spectra with data library Wiley 7N (Wiley Registry™ of Mass Spectral Data, 7th ed., USA) and retention times of these compounds in previously published data.

4.9 Protein oxidation

4.9.1 Carbonyl content (studies II-III)

The carbonyl content was determined by the DNPH assay with minor modifications (Soglia et al. 2016). Briefly, 400 µL of emulsion was treated with 0.8 mL of 0.3% (w/v) DNPH (Sigma-Aldrich) in 3 M HCl, or 3 M HCl (as blank) for 30 min. Then, the mixture was precipitated by 400 µL of 40% TCA. The pellet was collected by centrifugation at 5000 × g (study II) and 10000 × g (study III) for 5 min, washed three times with ethanol:ethyl acetate (1:1 v/v), dried with nitrogen, and dissolved in 6.0 M CH_6ClN_3. Then, the absorbance was measured at 280 nm and 370 nm. The carbonyl content was calculated by the following equation:

$$Carbonyl\ content\ (nmol/mg\ protein) = \frac{Abs_{370} - Abs_{370}(blank)}{22000 \times \left[Abs_{280} - \left(Abs_{370} - Abs_{370}(blank)\right) \times 0.43\right]} \times 10^6$$

where 22000 is the molar extinction coefficient and 0.43 is the coefficient for removing potential hydrazine interference at 280 nm (Soglia et al. 2016).

4.9.2 Free sulfhydryl content (studies II-III)

Free sulfhydryl was determined using the Ellman's reagent with minor modifications (Ou et al. 2004). Briefly, 1 mL of emulsion was mixed with 5 mL of acetone and centrifuged at 3,000 × g for 15 min. The pellet was dried by nitrogen and then dissolved in 5 mL 0.1 M Tris-HCl (pH 8.0). Further centrifugation (500 × g, 5 min) was needed if there were undissolved particles. Then, the free sulfhydryl was determined based on the DTNB method (Ellman 1959).

4.9.3 Trp fluorescence (studies I-III)

The loss of Trp fluorescence was measured as another index for protein oxidation in emulsions (Estevez et al. 2008). It was measured by a PerkinElmer LS 55 Luminescence Spectrometer using excitation of 295 nm. The Trp fluorescence emission spectra were recorded in the range of 310 and 400 nm, with slit width of 7 nm and speed of 180 nm/min. In study II, the Trp fluorescence of adsorbed or unadsorbed proteins were measured as well using the same parameters.

4.10 Statistical analysis

Statistical data analysis was conducted on SAS software (version 9.4, SAS Institute, Cary, NC, USA). To compare treatment effects among groups, one-way analysis of variance (ANOVA) and Tukey's post hoc testing was used. To compare time-dependent changes in each group, repeated measure (RM) One-way ANOVA with Tukey's or Dunnett's tests was used. Data were expressed as mean ± standard deviation (SD), and the significant level was set at $\alpha = 0.05$. In all studies, the modification of FBPI for each condition was performed once, and the resulting products were used to prepare three batches of emulsions at each condition. Other analyses were carried out with three replicates.

5 Results

5.1 MW changes of FBPI after enzymatic treatments (studies I-II)

Enzymatic treatments by either MTG or Alcalase caused significant changes in the FBPI MW, indicated by the changes of main bands in SDS-PAGE. The main bands of native-FBPI were MWs of ~22 kDa, ~37 kDa, ~42 kDa, and ~50 kDa (**Figure 7**, lane 1 of **A** and lane 1 of **B**). Both controls in study I (MTG0-FBPI) and study II (DH0-FBPI) showed similar pattern with their corresponding native FBPI, indicating the inactivation of enzymes was thorough and the amount of enzyme did not affect the SDS-PAGE pattern (**Figure 7**, lane 2 of **A** and lane 2 of **B**). In study I, MTG treatment generated new protein bands which corresponded to MWs ~65 kDa, ~85 kDa, and >118 kDa (**Figure 7,** lane 3-5 of **A**). In study II, Alcalase treatment effectively cleaved FBPI. DH of 4% noticeably reduced the main bands, with concomitantly increasing appearance of protein bands with MW of <18 kDa (**Figure 7**, lane 3 of **B**). Further hydrolysis with DHs of 9 and 15% produced bands that were all under MW of 15 kDa (**Figure 7**, lane 4 and 5 of **B**).

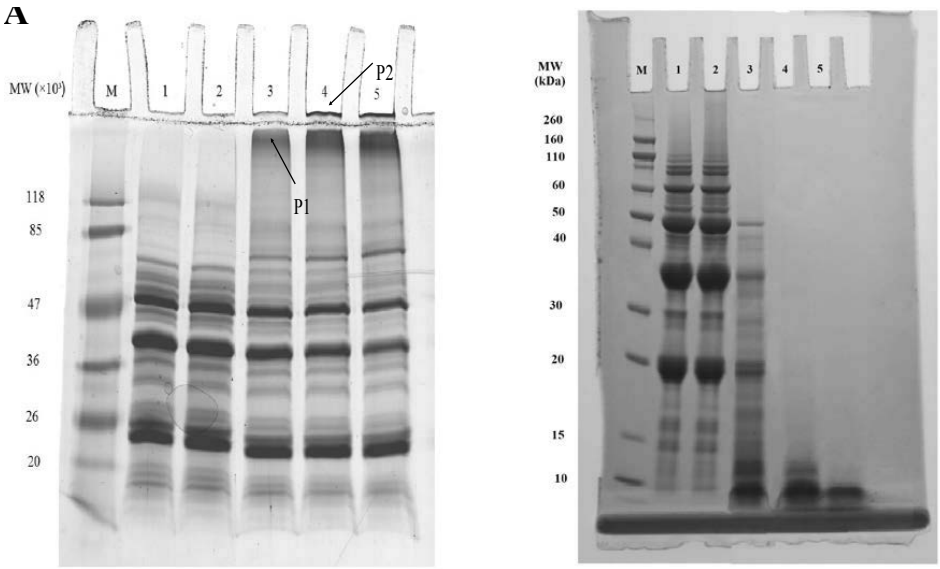

Figure 7. (A): SDS-PAGE patterns of study I. Lanes: M = protein marker (in kDa); 1 = native-FBPI; 2 = MTG0-FBPI (control-FBPI); 3-5 = MTG60-FBPI, MTG120-FBPI and MTG240-FBPI. P1 and P2 were new polymeric species formed under MTG treatment. FBPI was incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60-FBPI), 120 min (MTG120-FBPI), or 240 min (MTG240-FBPI). (B): SDS-PAGE patterns of study II. Lanes: M = protein marker (in kDa); 1 = native-FBPI; 2 = DH0-FBPI (control-FBPI); 3-5 = DH4-FBPI, DH9-FBPI, and DH15-FBPI. FBPI was incubated with alcalase (0.01 AU/g protein) at 50 °C and pH of 8.0 with DH of 4% (DH4-FBPI), 9% (DH9-FBPI), and 15% (DH15-FBPI).

5.2 Emulsifying properties of modified FBPIs

5.2.1 Surface charge (studies I-III)

The control-FBPI in the three studies showed different surface charges due to different environmental pH. With a pI of ~ 4.5, FBPI was negatively charged with ~ 28.7 mV and ~ 41.9 mV at pH 7.0 (study I) and 8.0 (study II), respectively, while it became positively charged with ~ 35.2 mV at pH 3.0 (study III) (**Figure 8**). Modifications of FBPI induced significant changes in its surface charge. MTG treatment induced cross-linking, which decreased the zeta potential gradually by 5-8% at pH 7 while hydrolysis by Alcalase induced continuously increased electronegativity, up to 22% for DH15-FBPI. On the other hand, when FBPI formed a soluble complex with CH at pH 4.8 via electrostatic interaction, the electronegativity of CH was decreased from ~35.3 mV (only CH) to ~27.8 mV (soluble complex) by negatively charged FBPI at pH 4.8.

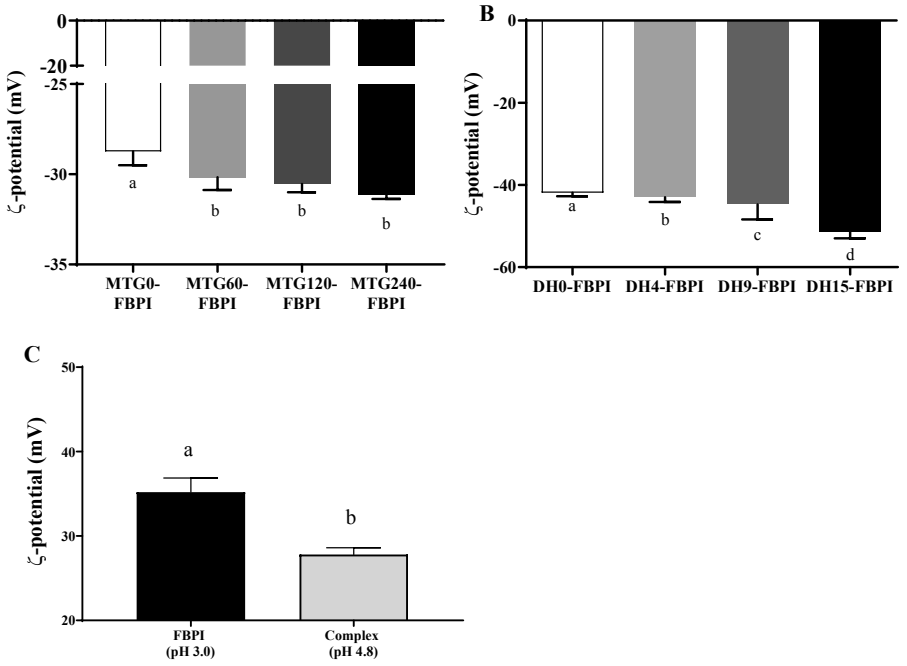

Figure 8. (A): Zeta potential of MTG0-FBPI (control-FBPI) and MTG-FBPIs in study I. FBPI was incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60-FBPI), 120 min (MTG120-FBPI), or 240 min (MTG240-FBPI) (n=3); (B): zeta potential of DH0-FBPI (control-FBPI) and DH-FBPIs in study II. FBPIs were incubated with Alcalase (0.01 AU/g of protein) at 50 °C and pH of 8.0 with DHs of 4% (DH4-FBPI), 9% (DH9-FBPI), and 15% (DH15-FBPI) (n=3); (C): zeta potential of FBPI (pH 3.0) and soluble FBPI-CH complex (pH 4.8) solutions in study III. The soluble FBPI-CH complex solution was prepared by mixing FBPI and CH solution at 1:1 (v/v) (n=3). Significant differences ($p < 0.05$) were denoted by different letters.

5.2.2 Surface hydrophobicity (studies I-III)

In study I, cross-linking by MTG treatment for 120 and 240 min, but not for 60 min increased the surface hydrophobicity by 13% and 8%, respectively. The effects of hydrolysis by Alcalase treatment on surface hydrophobicity depended on the DHs in that DH of 4% led to an 18% increase, while DHs of 9% and 15% resulted in markedly decreased hydrophobicity. In study III, binding to CH via electrostatic interaction remarkably decreased the surface hydrophobicity compared to native FBPI (**Figure 9**).

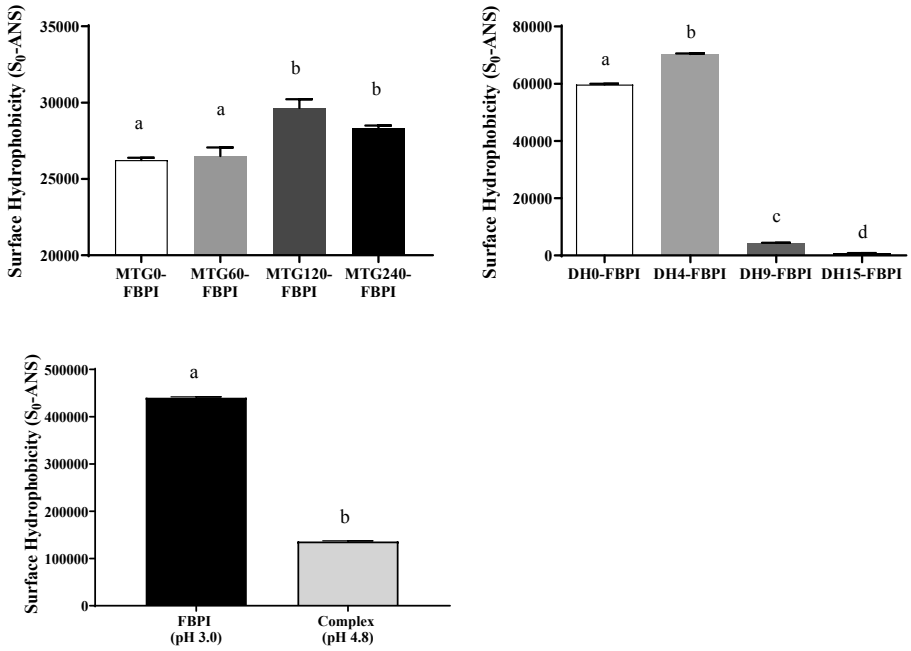

Figure 9. (A): Hydrophobicity of MTG0-FBPI (control-FBPI) and MTG-FBPIs in study I. FBPI was incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60-FBPI), 120 min (MTG120-FBPI), or 240 min (MTG240-FBPI) (n=3); (B): hydrophobicity of DH0-FBPI (control-FBPI) and DH-FBPIs in study II. FBPIs were incubated with Alcalase (0.01 AU/g of protein) at 50 °C and pH of 8.0 with DHs of 4% (DH4-FBPI), 9% (DH9-FBPI), and 15% (DH15-FBPI) (n=3); (C): hydrophobicity of FBPI (pH 3.0) and soluble FBPI-CH complex (pH 4.8) solutions in study III. The soluble FBPI-CH complex solution was prepared by mixing FBPI and CH solution at 1:1 (v/v) (n=3). Significant differences ($p < 0.05$) were denoted by different letters.

5.2.3 Protein solubility (studies I-II)

MW is an important factor affecting solubility. Therefore, cross-linking and hydrolysis were expected to have different effects on FBPI solubility. In study I, despite cross-linking by MTG modification, the solubility of FBPI did not change significantly. In study II, Alcalase hydrolysis improved the FBPI solubility by 6-10% at pH 8. In agreement with reduced MW

and increased electronegativity in study II, the highest solubility was found with DH15-FBPI (82.6 ± 2.0%) that exhibited smallest MW and highest electronegativity.

5.2.4 Interfacial tension (study III)

In study III, the interfacial tensions of FBPI (pH 3.0) and soluble FBPI-CH complex (pH 4.8) solutions were determined. They showed similar interfacial tension values of around 42 mN/m (**Figure 10**). In addition, the solutions of FBPI & CH mixture (pH 3.0) also showed similar interfacial tension as FBPI (data not shown), suggesting that free CH in aqueous phase had no further effect on lowering interfacial tension when FBPI served as emulsifier.

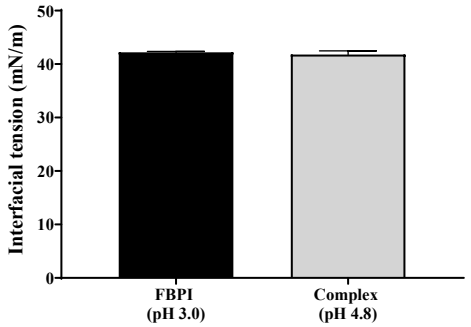

Figure 10. Interfacial tension of FBPI (pH 3.0) and soluble FBPI-CH complex (pH 4.8) solutions in study III. The soluble FBPI-CH complex solution was prepared by mixing FBPI and CH solution at 1:1 (v/v) (n=3).

5.3 Interfacial layer properties

5.3.1 Protein adsorption fractions (F_{ads}) (studies II-III)

In study II and III, the protein adsorption fractions (F_{ads}, %) in emulsions were measured during the oxidative course. In study II, the emulsion emulsified by moderately hydrolyzed FBPI (DH4) tended to have a 12% higher F_{ads} value than emulsion by control-FBPI (DH0) (p = 0.06) on day 0, suggesting increased surface coverage at the interfacial layer. Extensively hydrolysis might facilitate the adsorption of FBPI during preparation of emulsions in that DH15 emulsion exhibited the highest F_{ads} value at the beginning (**Table 4**). However, the F_{ads} values in DH9 and DH15 emulsions were decreased by 12% and 17% by day 7, suggesting a dramatic release of proteins from the oil-water interface during storage. The surface load (Γ_s) based on particle size of emulsion was further calculated. In comparison to FBPI hydrolysates with DHs of 4 and 0%, which had similar Γ_s of ~4 mg/m^2, hydrolysates with DHs of 9% and 15% had Γ_s of ~10 mg/m^2, indicating relatively lower emulsifying capacity (**Figure 11**).

Table 4. Protein adsorption fractions (F_{ads}, %) in emulsions of study II prepared by FBPI hydrolysates (Mean ± SD)[1].

Time	F_{ads} (%)			
(day)	DH0	DH4	DH9	DH15
0	$20.0 \pm 1.1^{a\ A}$	$22.4 \pm 0.9^{ab\ A}$	$18.7 \pm 1.4^{a\ A}$	$27.0 \pm 1.8^{b\ A}$
1	$26.9 \pm 0.6^{a\ B}$	$20.2 \pm 2.5^{c\ A}$	$9.5 \pm 1.2^{d\ B}$	$24.8 \pm 1.7^{ab\ A}$
4	$21.7 \pm 12.2^{a\ AB}$	$22.4 \pm 10.8^{a\ A}$	$3.2 \pm 0.9^{c\ B}$	$10.2 \pm 1.7^{b\ B}$
7	$28.5 \pm 10.0^{a\ AB}$	$21.7 \pm 9.0^{a\ A}$	$6.5 \pm 1.9^{b\ B}$	$9.8 \pm 1.7^{b\ B}$

[1] DH0, DH4, DH9, and DH15 emulsions were prepared with 1% (w/v) DH0-FBPI or hydrolyzed FBPIs with DHs of 4, 9, and 15% and stored at 37 °C in the dark for 7 days (n=3). Different lowercase letters indicated significant group differences ($p < 0.05$) on each day, determined by one-way ANOVA with Tukey's test (n=3). Different capital letters indicated significant differences among different days within each group, determined by RM one-way ANOVA with Tukey's test ($p < 0.05$).

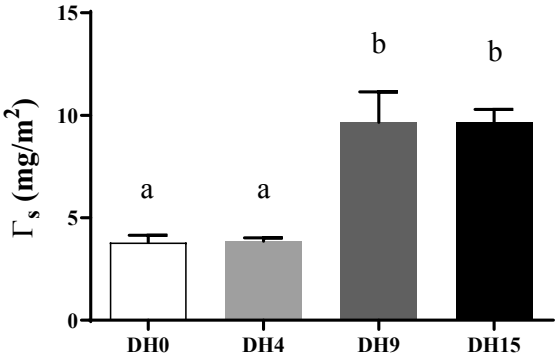

Figure 11. Surface load (Γ_s) in emulsions of study II stored at 37 °C in the dark for 7 days. DH0, DH4, DH9, and DH15 emulsions were stabilized by 1% (w/v) DH0-FBPI or hydrolyzed FBPIs with DHs of 4, 9, and 15% and stored at 37 °C in the dark for 7 days (n=3). Significant differences ($p < 0.05$) were denoted by different letters.

In study III, the initial F_{ads} in the four emulsions was in the order of EML > EMC > EMFB > EMN (**Figure 12**). Noticeably, the F_{ads} in EML was increased continuously compared to others during storage. In contrast, the F_{ads} in EMFB and EMN were much lower during the whole process. The calculation of Γ_s was not applicable as a result of specific structure of EML and EMC, thus was not employed in study III.

5.3.2 Interfacial layer thickness and configuration (study III)

The layer thicknesses of EMFB, EMN, EML, and EMC were determined using uniform polystyrene latex particles as adsorption surface (**Figure 13A**). EMFB and EMN had similar layer thickness of ~ 19.1 nm, which was significantly lower than EML (66.0 ± 0.7 nm) and EMC (57.5 ± 0.5 nm). EML had moderately thicker layer than EMC. The potential

configurations of CH and FBPI at the interfacial layer were illustrated schematically illustrated in **Figure 13B** (CH and FBPI were represented in green and red, respectively; the size of oil droplet, FBPI, and CH might be not to scale).

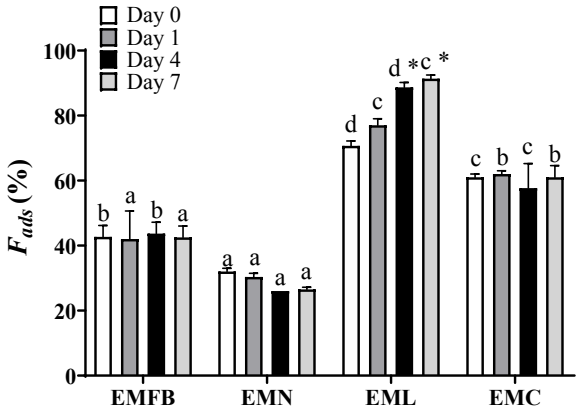

Figure 12. Protein adsorption fraction (F_{ads}) of emulsions in study III. All the emulsions contained 5% (w/v) oil, 0.5% (w/v) FBPI, and 0.02% (w/v) sodium azide. EMC, EML and EMN contained 0.05% (w/v) CH. All emulsions were stored at 37 °C in dark for 7 days (n=3). Different letters indicated significant group differences on each day, determined by one-way ANOVA with Tukey's test ($p < 0.05$). (∗) Significant differences ($p < 0.05$) from day 0 within each group, determined by RM one-way ANOVA with Dunnett's test.

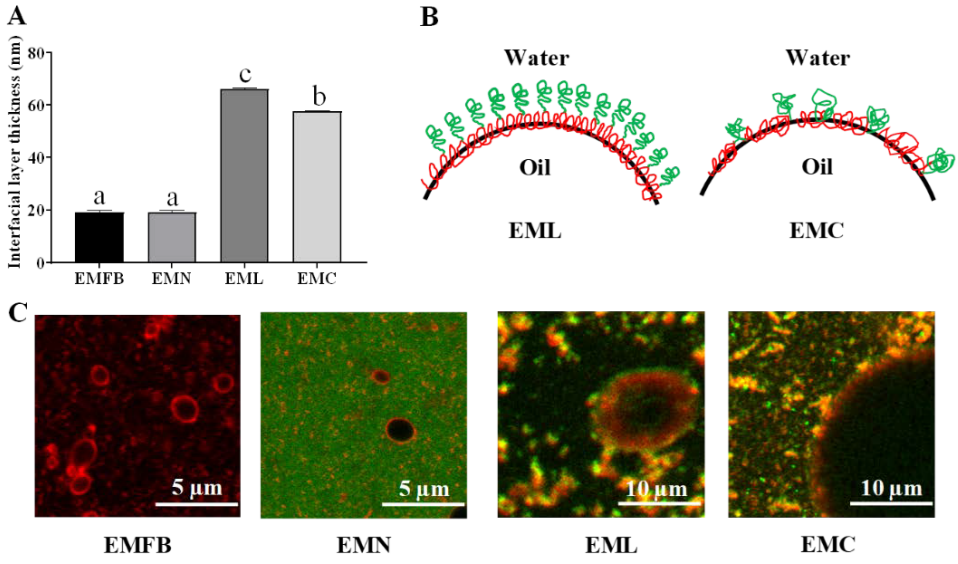

Figure 13. (A) Adsorbed layer thickness of emulsions, (B) schematic presentations of structure of interfacial layer in EML and EMC, and (C) representative confocal laser scanning microscope pictures of fresh emulsions in study III. All the emulsions contained 5% (w/v) oil, 0.5% (w/v) FBPI, and 0.02% (w/v) sodium azide. EMC, EML and EMN contained 0.05% (w/v) CH. All emulsions were stored at 37 °C in dark for 7 days (n=3). Significant differences ($p < 0.05$) were denoted by different letters.

5.3.3 Visualization of interfacial layer structure (study III)

CLSM was employed for further visualization of FBPI and CH distribution at the interfacial layer and in the aqueous phase of emulsions (**Figure 13C**). CH, FBPI, and oil droplets were represented in green, red, and black, respectively. In EMFB and EMN, a thin bright red circle covered around the oil droplet, which corresponded to mono-FBPI layer. In EMN, green color was uniformly dispersed in the aqueous part, indicating no visible interaction between CH and FBPI in either aqueous or interfacial phase. In EMC, the oil droplet was covered by a thicker and mixed colored (red and green) layer. Part of labelled complex appeared in the aqueous phase as well. In EML, the oil droplet was surrounded by an inner red circle and an outer green circle. The yellow-like color somewhere between might represent color overlay. In addition, the interfacial layer of EML appeared to be more compact and denser than that in EMC.

5.3.4 Low-shear viscosity (study III)

Due to the relatively low concentration of CH (0.05%) in emulsions and the low viscosity obtained in corresponding solutions (data not shown), the viscosity-stabilizing effect of CH in the aqueous phase is negligible. EML and EMC had around 1.4-fold higher low-shear viscosity than that of EMFB and EMN (**Figure 14**).

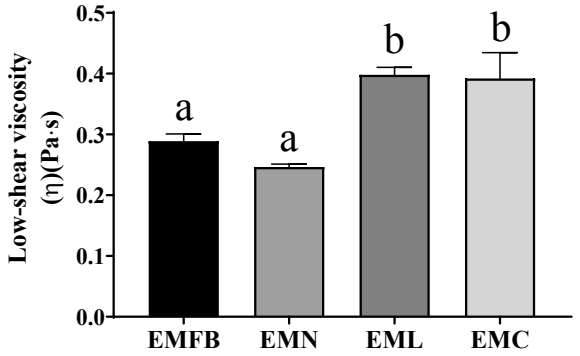

Figure 14. Low-shear viscosity of emulsions in study III. All the emulsions contained 5% (w/v) oil, 0.5% (w/v) FBPI, and 0.02% (w/v) sodium azide. EMC, EML and EMN contained 0.05% (w/v) CH. All emulsions were stored at 37 °C in dark for 7 days (n=3). Significant differences ($p < 0.05$) were denoted by different letters.

5.4 Physical stability of emulsions

5.4.1 Changes in droplet sizes (studies I-III)

In study I, emulsions emulsified by MTG-modified FBPIs had greater droplet sizes than MTG0 (**Figure 15**). The $d_{3,2}$ of MTG0 and MTG-FBPIs emulsions ranged from 0.06 to 0.15 μm on day 0. MTG treatment for 60, 120, and 240 min increased the $d_{3,2}$ values by 19%, 59%, and 135%, respectively.

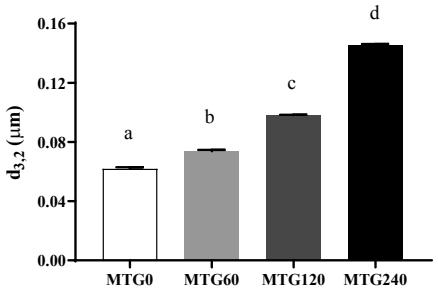

Figure 15. The surface mean diameter ($d_{3,2}$) of MTG0, MTG60, MTG120, and MTG240 on day0 in study I. Emulsions were emulsified with 3% FBPI that were incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60), 120 min (MTG120), or 240 min (MTG240); emulsion stabilized with 3% FBPI that was incubated with NH_4Cl-inactivated MTG served as control. Significant differences ($p < 0.05$) were denoted by different letters (n=3).

In study II, the effects of Alcalase treatment on droplet size were dependent on the DHs. On day 0, the levels of $d_{3,2}$ in DH9 and DH15 were much larger than that in DH0 and DH4 (**Table 5**). From day 0 to day 7, the levels of $d_{3,2}$ of DH9 and DH15 emulsions greatly increased by more than 10-fold. In contrast, the levels of $d_{3,2}$ in DH0 and DH4 emulsions were maintained during 7 days of storage.

Table 5. The surface mean diameter of emulsions in study II, prepared by control FBPI or hydrolyzed FBPIs (Mean ± SD)[1].

Time	$d_{3,2}$ (nm)			
(Day)	DH0	DH4	DH9	DH15
0	$57 \pm 3^{a\,A}$	$52 \pm 1^{a\,A}$	$155 \pm 12^{b\,A}$	$107 \pm 1^{b\,A}$
1	$117 \pm 1^{a\,A}$	$71 \pm 1^{a\,A}$	$1240 \pm 185^{b\,B}$	$1363 \pm 45^{b\,B}$
4	$116 \pm 4^{a\,A}$	$144 \pm 27^{a\,A}$	$1679 \pm 12^{b\,B}$	$1603 \pm 52^{b\,B}$
7	$234 \pm 70^{a\,A}$	$111 \pm 16^{a\,A}$	$1568 \pm 332^{b\,B}$	$2097 \pm 1480^{b\,B}$

[1] DH0, DH4, DH9, and DH15 emulsions were prepared with 1% (w/v) of control FBPI or hydrolyzed FBPIs with DH of 4%, 9%, and 15% and stored at 37 °C in dark for 7 days (n=3). Different lowercase letter indicated significant group differences ($p < 0.05$) on each day, determined by One-way ANOVA with Tukey's test (n=3). Different uppercase letter indicated significant differences ($p < 0.05$) among different days within each group, determined by RM One-way ANOVA with Tukey's test.

In study III, together with $d_{3,2}$, the volume mean diameter ($d_{4,3}$) was recorded as well. On Day 0, the levels of $d_{3,2}$ in EMC and EML were larger than that in EMFB (**Figure 16 A)**. In addition, EML had ~8% smaller $d_{3,2}$ than EMC. During accelerated storage at 37 °C, EMFB, EMN, and EML exhibited monomodal distribution (data not shown) with steadily increased $d_{3,2}$ droplet size while EMC showed bimodal distributions on day 1 and 7 (**Figure 16C**, take day 1 as an example). Correspondingly, EMC had spikes in $d_{4,3}$ on day 1 and 7 (**Figure 16B**).

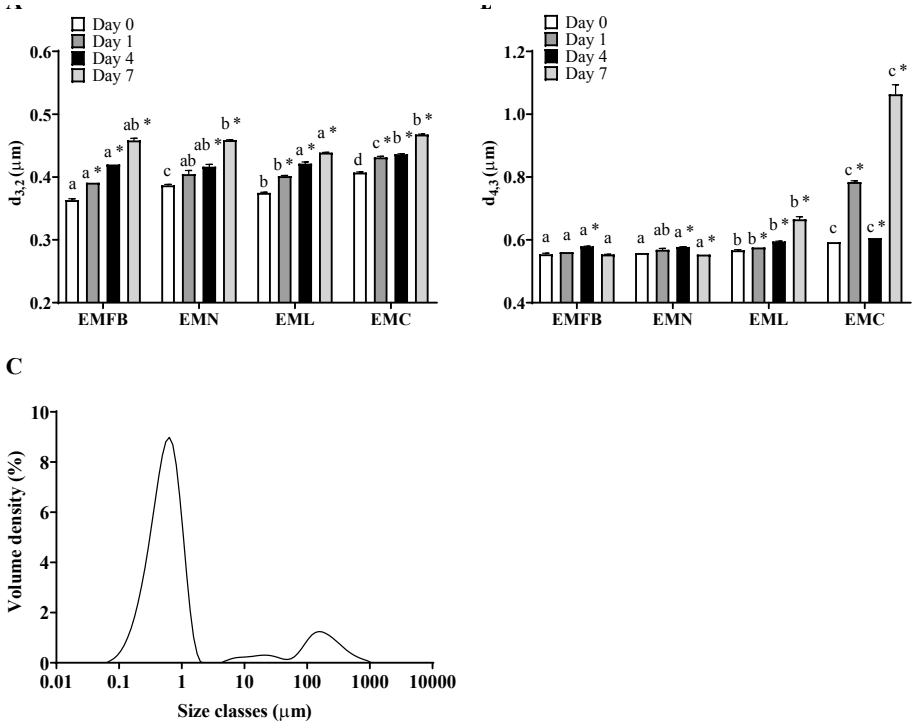

Figure 16. Particle size of emulsions (A) $d_{3,2}$ and (B) $d_{4,3}$ in study III; (C) representative particle size distribution of EMC on day 1. All the emulsions contained 5% (w/v) oil, 0.5% (w/v) FBPI, and 0.02% (w/v) sodium azide. EMC, EML and EMN contained 0.05% (w/v) CH. All emulsions stored at 37 °C in the dark for 7 days (n=3). Different letters indicated significant group differences on each day, determined by one-way ANOVA with Tukey's test ($p < 0.05$). (∗) Significant differences ($p < 0.05$) from day 0 within each group, determined by RM one-way ANOVA with Dunnett's test.

5.4.2 Microscopy

The changes in particle morphology in emulsions were observed during storage and compared with changes in particle size to further assess physical stability of emulsions.

In study I, microscopy revealed that emulsions prepared by MTG-FBPIs had larger initial droplets than the MTG0 (**Figure 17**), which was in accordance with the $d_{3,2}$ measurements. The MTG0 and MTG60 emulsions exhibited more homogeneous distribution than MTG120

and MTG240 emulsions. After 7 days of storage, the MTG0 and MTG60 emulsions appeared stable while droplets coalescence was observed in MTG120 and MTG240 emulsions.

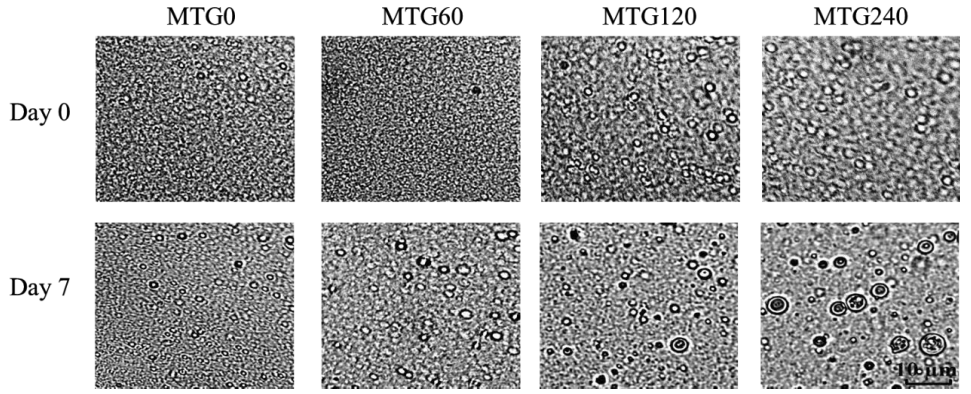

Figure 17. The representative microscopic pictures of emulsions on day 0 day 7 in study I. Emulsions were prepared with 3% FBPI that were incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60), 120 min (MTG120), or 240 min (MTG240); emulsion prepared with 3% FBPI that was incubated with NH_4Cl-inactivated MTG served as control. All emulsions were stored at 37 °C in dark for 7 days.

In study II, DH0 and DH4 emulsions displayed homogeneous distribution on the first day and appeared to remain relatively stable after 7 days of storage. In contrast, visible droplet coalescence was observed in DH9 and DH15 emulsions after 7 days (**Figure 18**).

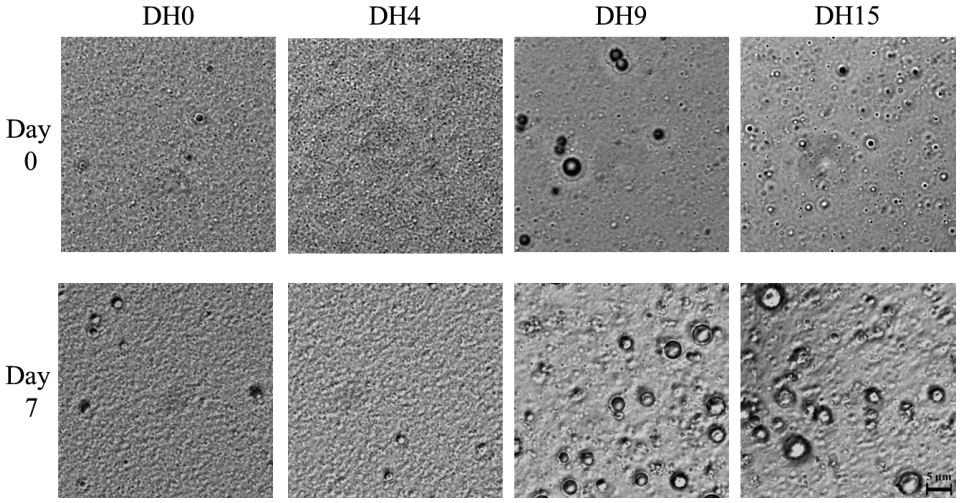

Figure 18. The representative microscopic pictures of emulsions on day 0 and 7 in study II. DH0, DH4, DH9, and DH15 emulsions were prepared with 1% (w/v) of control or hydrolyzed FBPIs with degree of hydrolysis (DH) of 4%, 9%, and 15%, and stored at 37 °C in dark for 7 days.

In study III, microscope observation showed slight flocculation or coalescence in EMC since day 1 (**Figure 19**), which agreed well with the $d_{4,3}$ results. In EMN, bigger oil droplets were observed on day 1. Comparably, the homogeneity of EMFB and EML were maintained during 7 days of storage.

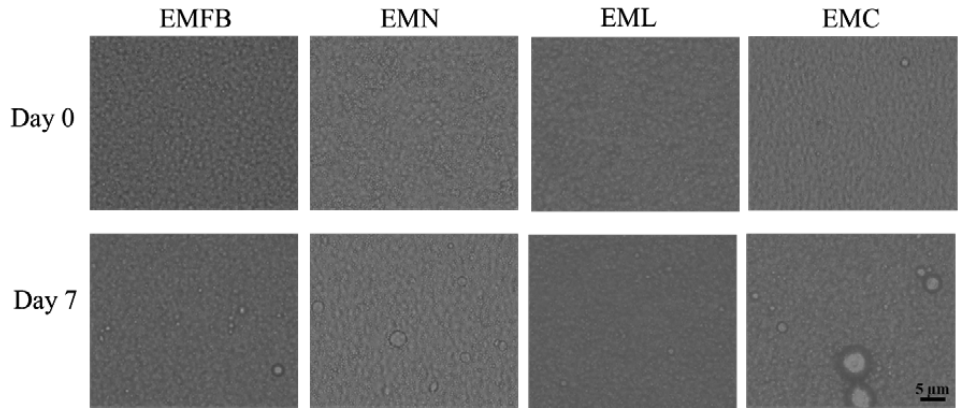

Figure 19. The representative microscopic pictures of emulsions on days 0, 1, 4, and 7 in study III. All the emulsions contained 5% (w/v) oil, 0.5% (w/v) FBPI, and 0.02% (w/v) sodium azide. EMC, EML and EMN contained 0.05% (w/v) CH. All emulsions were stored at 37 °C in dark for 7 days.

5.4.3 ΔBS and TSI (studies II-III)

To better characterize the phenomena of destabilization, Turbiscan backscattering (BS) data were plotted against sample height over time.

In Study II, taking the DH9 emulsion as an example (**Figure 20A**), the apparent deviations among the scans at the same time point on different days were observed. The level of ΔBS continued to decrease during storage, suggesting increased particle size as a result of flocculation or coalescence. Meanwhile, the ΔBS signal was increased at the top of the sample vial, suggesting that a concomitant creaming took place. Further, the changes in ΔBS and sample height were synthesized into the TSI which reflects the destabilization of emulsions by summing up variations including creaming, coalescence, and/or flocculation. In comparison to the DH9 and DH15 emulsions, DH0 and DH4 emulsions showed much lower TSI values during 7 days of storage, indicating better physical stability (**Figure 20B**).

In study III, taking the EMC emulsion as an example (**Figure 21A**), EMC exhibited the lowest physical stability indicated by apparent decreases in ΔBS between 0 to 15 cm height in sample vials. This suggested partial flocculation or coalescence-induced sedimentation in

EMC during storage. The emulsion physical stabilities were further compared by calculating the TSI during storage (**Figure 21B**). TSI in EMC was increased continuously and reached the highest at day 7, indicating least physical stability. In EMN, bigger oil droplets were observed on day 1, which corresponded to the notably high TSI value. However, it was not reflected by either $d_{3,2}$ or $d_{4,3}$. The relatively low stability in EMN agreed well with the decreasing F_{ads} during storage.

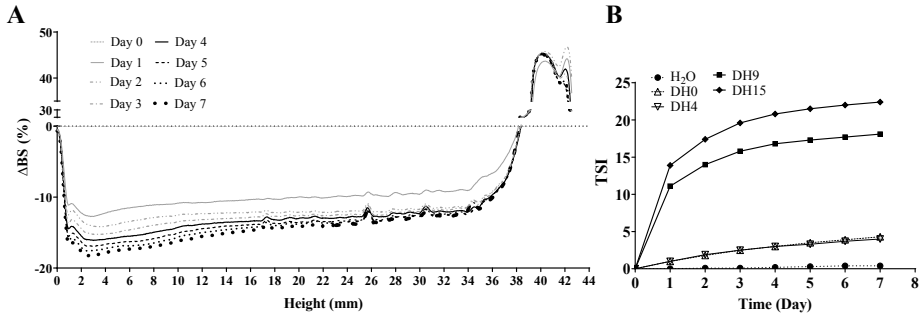

Figure 20. (A) ΔBS in DH9 emulsion, (B) TSI in all emulsions of study II stored at 37 °C in the dark for 7 days. DH0, DH4, DH9, and DH15 emulsions were prepared by 1% (w/v) control FBPI or hydrolyzed FBPIs with DHs of 4, 9, and 15% and stored at 37 °C in the dark for 7 days.

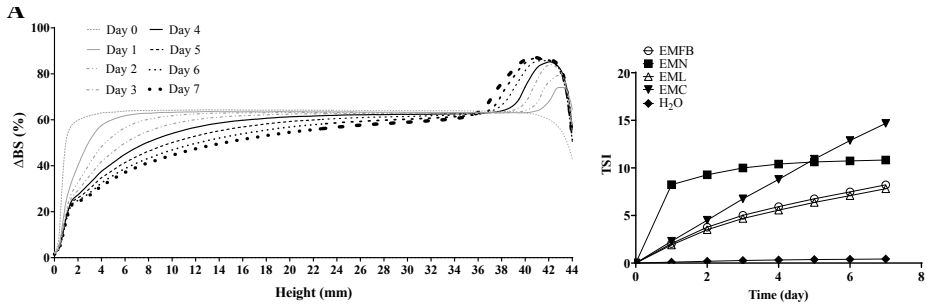

Figure 21. (A) ΔBS of EMC, and (B) TSI in all emulsions stored at 37 °C in the dark for 7 days. All the emulsions contained 5% (w/v) oil, 0.5% (w/v) FBPI, and 0.02% (w/v) sodium azide. EMC, EML and EMN contained 0.05% (w/v) CH.

5.5 Oxidative stability of emulsions (studies I-III)

5.5.1 Lipid oxidation

In study I, both CDs and hexanal levels continuously increased in all emulsions (**Figure 22**). On day 7, CDs in MTG60, MTG120, and MTG240 emulsions were 17%, 14%, and 14% lower than MTG0. Similarly, hexanal in MTG60 and MTG120 was 65% and 62% lower than MTG0. In contrast, hexanal in MTG240 did not differ from MTG0. This indicated that

MTG treatment on FBPI for 60 and 120 min, but not 240 min increased the oxidative stability of lipids in O/W emulsions.

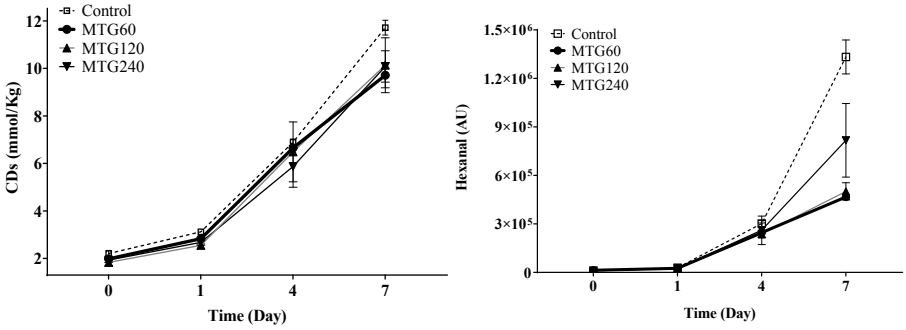

Figure 22. Formation of conjugated dienes (CDs) and hexanal in emulsions of study I stored at 37 °C in dark with constant magnetic stirring over 7 days (n=3). Emulsions were prepared with 3% FBPI that were incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60), 120 min (MTG120), or 240 min (MTG240); MTG0 prepared with 3% FBPI that was incubated with NH_4Cl-inactivated MTG served as control.

Similar to study I, the progression of lipid oxidation in study II took place in all emulsions. The levels of CDs were all above the baseline after 4 days of storage and reached a maximum at day 7 with increases of 1.9-4.0-fold (**Figure 23A**). DH4 emulsion displayed significantly lower CDs on days 4 and 7 compared to DH0 emulsion. In contrast, DH9 and DH15 had a similar or greater level of CDs than DH0 and DH4 emulsions. The formation of hexanal showed similar trends as CDs, with DH4 emulsion being the least oxidized (**Figure 23B**).

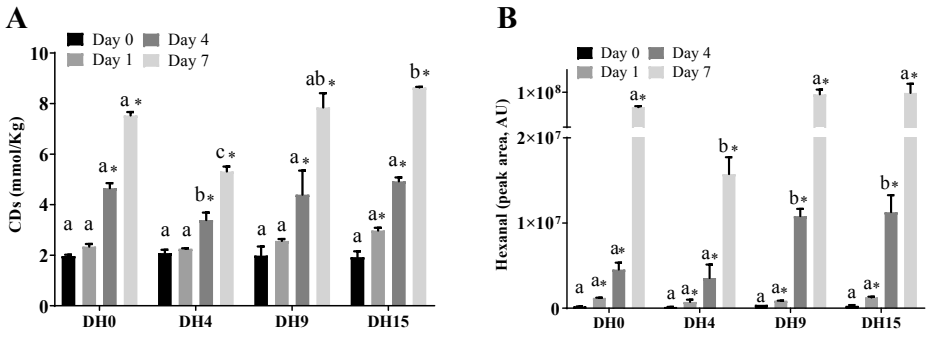

Figure 23. (A) CDs and (B) hexanal in emulsions of study II. DH0, DH4, DH9, and DH15 emulsions were prepared with 1% (w/v) control FBPI or hydrolyzed FBPIs with DHs of 4, 9, and 15% and stored at 37 °C in the dark for 7 days (n=3). Different letters indicated significant group differences on each day, determined by one-way ANOVA with Tukey's test ($p < 0.05$). (∗) Significant differences ($p < 0.05$) from day 0 within each group, determined by RM one-way ANOVA with Dunnett's test.

In study III, lipid oxidation developed in all emulsions as well. Formation of primary lipid oxidation product CDs during storage increased continuously (**Figure 24A**). On day 1 and 4, EML had the lowest CDs of all. By day 7, EML had lower CDs than EMFB. The CDs in EMN and EMC showed a decrease. To further determine lipid oxidation, secondary oxidation products from oleic acid, linoleic acid or linolenic acid in rapeseed oil, including hexanal, 2-octenal, 2-heptenal, 2-pentenal, 2-pentylfuran, 2,4-heptadienal, 2-hexenal, and heptanal were measured (**Figure 24B-I**). These volatile compounds in all emulsions were gradually produced and reached the highest level at the end of storage. In general, the secondary oxidation products were lower in EML than in EMFB. EMC did not show better lipid oxidative stability despite its thicker interfacial layer, as EML. It is noteworthy that EMN showed the highest lipid oxidation during storage.

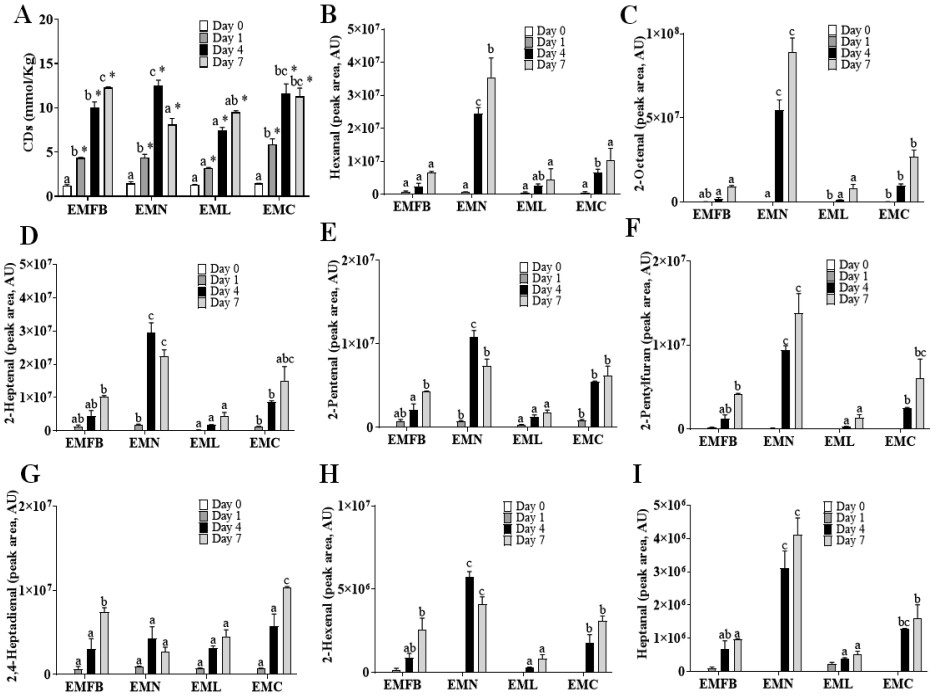

Figure 24. Primary and secondary lipid oxidation products (A) CDs, (B) hexanal (C) 2-octenal, (D) 2-heptenal, (E) 2-pentenal, (F) 2-pentylfuran, (G) 2,4-heptadienal, (H) 2-hexenal and (I) measured in emulsions of study III. All the emulsions contained 5% (w/v) oil, 0.5% (w/v) FBPI, and 0.02% (w/v) sodium azide. EMC, EML and EMN contained 0.05% (w/v) CH. All emulsions were stored at 37 °C in the dark for 7 days (n=3). Different letters indicated significant group differences on each day, determined by one-way ANOVA with Tukey's test ($p < 0.05$). (∗) Significant differences ($p < 0.05$) from day 0 within each group, determined by RM one-way ANOVA with Dunnett's test.

5.5.2 Protein oxidation

In study I, MTG120 and MTG240 emulsions started to show significant lower Trp FI than MTG0 emulsion on day 1 (**Figure 25 A**). By contrast, MTG60 emulsion maintained similar level of Trp FI with MTG0 emulsion till day 4. To better understand why longer MTG treatment on FBPI appeared to accelerate protein oxidation, emission spectra of Trp fluorescence was recorded (**Figure 26**). Fluorescence peak of Trp in MTG-FBPIs emulsions had a red shift from ~330 nm to ~334 nm. Besides Trp, the MTG-FBPIs emulsions had similar level of protein oxidation products (OPs) (mainly refers to carbonyl compounds) with MTG0 emulsions on day 1 and 4. On day 7, OPs in MTG60 and MTG120 were significantly higher than in the MTG0 emulsion (**Figure 25 B**). Thus, when the storage time was extended, MTG-FBPIs did not improve the oxidative stability of proteins in the O/W emulsions.

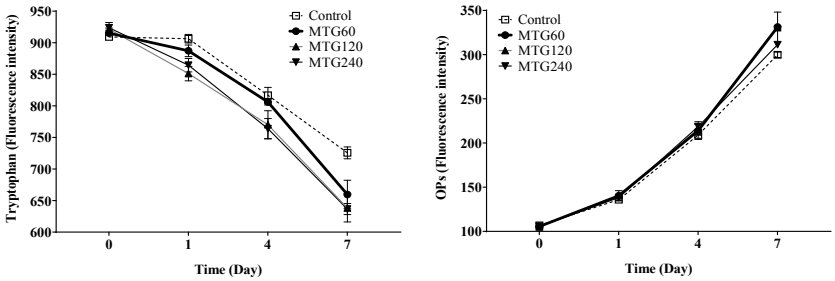

Figure 25. Fluorescence of Trp and protein oxidation products (OPs) in emulsions of study I stored at 37 °C in dark with constant magnetic stirring over 7 days (n=3). Emulsions were prepared with 3% FBPI that were incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60), 120 min (MTG120), or 240 min (MTG240); an emulsion prepared with 3% FBPI that was incubated with NH4Cl-inactivated MTG served as control.

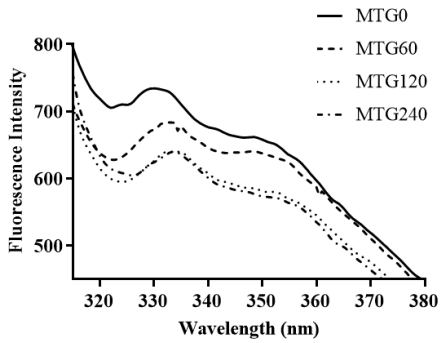

Figure 26. Changes of Trp FI in emulsions of study I incubated at 37 °C in dark with constant magnetic stirring over 7 days. Emulsions were prepared with 3% FBPI that were incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60), 120 min (MTG120), or 240 min (MTG240); emulsion stabilized with 3% FBPI that was incubated with NH4Cl-inactivated MTG served as control.

In study II, extensive hydrolysis induced more protein oxidation as DH9 and DH15 displayed higher levels of protein carbonyls, less free sulfhydryl groups, and lower Trp FI compared to DH0 (panels **A-C** of **Figure 27**). Although DH4 had greater initial protein oxidation than DH0, the progression of protein oxidation in DH4 was the slowest among all emulsions. By day 7, the carbonyl content increased by 56% in DH4, whereas there were 237, 132, and 71% increases in DH0, DH9, and DH15, respectively. In addition, DH4 had lower carbonyl but greater Trp content than DH9 and DH15, indicating better oxidative stability. We further determined Trp FI in adsorbed and unadsorbed proteins. It showed that the percentage of Trp oxidation in the aqueous phase was more pronounced than that in the interface in all emulsions (**Figure 27D**).

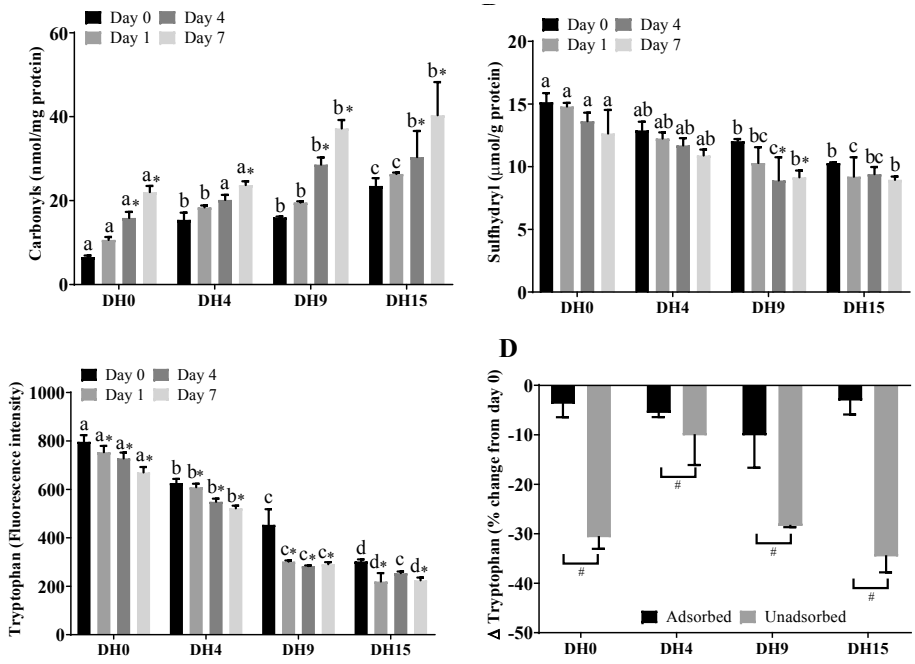

Figure 27. (A) Carbonyl content, (B) free sulfhydryl content, and (C) Trp FI in emulsions of study II. (D) percentage change of Trp FI from day 0 to day 7 in adsorbed and unadsorbed protein recovered by ultracentrifugation. DH0, DH4, DH9, and DH15 emulsions were prepared with 1% (w/v) control FBPI or hydrolyzed FBPIs with DHs of 4, 9, and 15% and stored at 37 °C in the dark for 7 days (n=3). Different letters indicated significant group differences on each day, determined by one-way ANOVA with Tukey's test ($p < 0.05$). (∗) Significant differences from day 0 within each group, determined by RM one-way ANOVA with Dunnett's test ($p < 0.05$). (#) Significant difference.

In study III, EML had noticeably less loss of Trp, and lower or similar level of carbonyls formation during storage compared to EMFB and EMN. It indicated less protein oxidation in EML (**Figure 28 A and B**). Despite the participation of CH in the interfacial layer in EMC,

the EMC generally showed slightly less loss of Trp but marginally more carbonyls formation than EMFB and EMN. On day 0, sulfhydryl concentration in EMC and EML was lower than EMFB. Sulfhydryl concentration was decreased continuously in EMFB. Interestingly, an increasing trend of sulfhydryl concentration was observed in EML and EMC (**Figure 28 C**).

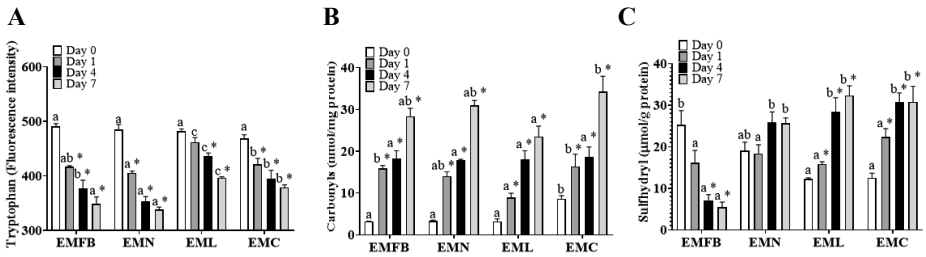

Figure 28. (A) Trp FI, (B) carbonyl content, and (C) free sulfhydryl content in emulsions of study III. All the emulsions contained 5% (w/v) oil, 0.5% (w/v) FBPI, and 0.02% (w/v) sodium azide. EMC, EML and EMN contained 0.05% (w/v) CH. All emulsions were stored at 37 °C in the dark for 7 days (n=3). Different letters indicated significant group differences on each day, determined by one-way ANOVA with Tukey's test ($p < 0.05$). (∗) Significant differences from day 0 within each group, determined by RM one-way ANOVA with Dunnett's test ($p < 0.05$).

6 Discussion

6.1 Modifications of MW by enzymatic treatments

Many studies have shown that food-grade enzymes can make profound changes in proteins, leading to improved texture and stability of food matrices (Luisa et al. 2015). MTGase was used to promote protein crosslinking in study I, while Alcalase was employed for hydrolysis reactions in study II. As shown by SDS-PAGE, FBPI is a good substrate for both MTGase and Alcalase resulting in significant changes of MW under optimum conditions.

MTGase has been widely applied in meat and dairy products, as a safe and effective way to modify proteins and obtain food products with enhanced or innovative textural characteristics (Luisa et al. 2015). MTGase mainly modifies proteins by affecting intra- and intermolecular ε-(γ-glutamyl)-lysine (G-L) cross-links, and both Lys and Gln residues are present in high amounts in FBPI (Hossain and Mortuza 2006). The wide distribution of Gln residues within FBPI allows MTGase to produce various cross-linked oligomers with different MWs by forming isopeptide bonds between two amino acid residues. It has been shown that MTGase influences on 11S and 7S of FBPI (MWs of ~22 kDa, ~37 kDa, ~42 kDa, and ~50 kDa) efficiently (Wright and Boulter 1972; 1974). The new protein species should be homo- and/or hetero-dimers and polymers of 11S and 7S by cross-linking (Damodaran and Agyare 2013). In addition, along with increased treatment time, new polymeric species (P1 and P2, >118 kDa) were aggregated at the top of separating gel and stacking gel, which appeared to be polymers from cross-linking between monomers and intermediary subunits of 11S and 7S (Wright and Boulter 1972; 1974). However, some electrophoretic bands of MTG treated FBPI showed resistance to the treatment suggesting that these peptides probably were in a much more compact structure. As a consequence, not all the Gln residue side chain groups were readily accessible by MTGase (Agyare et al. 2008). The production of new polymeric species (P1 and P2) with high-MW in MTGase-treated samples was extensive. This indicated that FBPI was a good substrate for MTGase. MTGase induced mostly intermolecular polymerization of FBPI rather than intramolecular (Damodaran and Agyare 2013).

Alcalase, which consists primarily of subtilisin A, is an industrial- and food-grade enzyme prepared from *Bacillus licheniformis*. It has been used extensively in various food applications due to its broader specificity and more affordable cost compared with other enzymes (Adamson and Reynolds 1996). Alcalase can cleave peptide bonds on the carboxyl side of Glu, Met, Leu, Tyr, Lys, and Gln that all together account for ~ 40% of total amino

acids in FBPI (Hossain and Mortuza 2006). Thus, Alcalase effectively broke down FBPIs into small MWs in study II, even when DH was only 4%.

The MWs of FBPI were significantly modified via either protein crosslinking (study I) or hydrolysis reactions (study II). As a result, the hydrophobicity, solubility, and surface charges of FBPI were altered, which in turn had an effect on its emulsifying properties.

6.2 Altered physiochemical properties of FBPI

Emulsifying property of proteins is strongly correlated with various physiochemical properties such as surface charge, hydrophobicity, solubility, and interfacial tension (Nakai et al. 1980). These physicochemical properties govern the behavior of proteins in P-O/W EMs and were discussed below.

6.2.1 Effects of enzymatic modifications

MTG treatment for 60 min slightly increased the hydrophobicity of FBPI (study I) which might be due to some hydrophobic residues in FBPI being partially exposed in cross-linked molecules as a result of peptide-peptide association. This was supported by the intrinsic fluorescence spectra of FBPI and MTGase-treated FBPIs. The fluorescence spectra of FBPIs were mostly contributed by Trp residue showing that the MTGase treatment altered the FI and position of Trp in FBPI. The increased FI and red shift in λ_{max} to a longer wavelength reflected the exposure of some of the Trp residues to a more polar environment (Agyare et al. 2008). Surface hydrophobicity is an index for assessing protein emulsifying functionality by measuring the capacity of proteins to facilitate intermolecular interactions (Nakai et al. 1980). A favorable balance of hydrophilic and hydrophobic groups is essential to make proteins ideal emulsifiers.

Limited MTG treatment also increased the electronegativity of FBPIs, which was attributed to the increase in negatively charged amino acids (Glu and Asp) due to deamidation (Agyare et al. 2008). Surface charge is critical for proteins to form and stabilize emulsions (Li and Tian 2002). Once a physical interfacial layer around the oil droplet is formed by proteins, the electrostatic charges of proteins produce repulsive forces that can prevent the coalescence (McClements et al. 2017). However, increased electronegativity did not promote increased solubility. It is possible that the effects of the increased electrostatic repel on solubility were offset by increased MWs and hydrophobicity due to cross-linking. As emulsifying activity is mainly influenced by protein solubility and hydrophobicity (Tavano 2013), FBPI with

limited MTGase treatment (MTG60-FBPI) exhibited similar emulsifying activity with control (MTG0-FBPI). However, extended cross-linking (MTG120-FBPI and MTG240-FBPI) induced decreased emulsifying activity, which may be partly explained by excessively increased MWs and improper surface hydrophobicity.

Low degree of hydrolysis (DH 4%) on FBPI increased the surface hydrophobicity (study II). This might be caused by greater exposure of embedded hydrophobic amino acid residues to the solvent (Xu et al. 2016). In turn, the increased hydrophobicity facilitated the adsorption of modified FBPI onto oil droplet surface. This was supported by the results of surface load (Γs) which corresponds to the mass of emulsifier required to cover a unit area of droplet surface (usually expressed as mg/m^2). The Γs measured in DH4 was smaller, which indicated that DH4-FBPI could cover greater oil-water interfacial area at a fixed lower amount. Thus, DH4-FBPI could serve as a better emulsifier. Meanwhile, the electronegativity of DH4-FBPI was also increased. This might be the result of increased peptides number and exposure of ionizable amino acids, according to Mahmoud et al. (1992). Paulson and Tung (1987) also proposed that the dissociation of carboxylic group at pH 8 by enzymatic hydrolysis could produce more carboxylate ions (COO^-), thus increasing electronegativity. The increase in net charge could, in turn, improve protein solubility due to the higher repulsive electrostatic force between the molecules. This was confirmed by the solubility test that showed higher solubility of DH4-FBPI than DH0-FBPI. Besides the effect of net charge on solubility, smaller peptides induced by hydrolysis also led to higher solubility because peptides with lower MWs could form stronger hydrogen bonds with water. This is in agreement with previous reports that enzymatic protein hydrolysis would induce increases in protein solubility (Tavano 2013). With smaller MWs and higher solubility, DH4-FBPI exhibited greater migration to the oil-water interface. Together, changes in these physiochemical characteristics made DH4-FBPI a better emulsifier than the native FBPI. However, extended hydrolysis leads unfavorable characteristics regarding emulsifying capacity. In this study, hydrolysis with DH of 9% and 15% decreased the surface hydrophobicity dramatically. It was mainly attributed to enzymatic breakdown of hydrophobic areas and reburying of exposed hydrophobic residues via hydrophobic interactions. Excess hydrolysis (DH of 9% and 15%) not only deprived FBPIs of the capacity to interact with both aqueous and non-aqueous phases, but also broke the balance of hydrophilic and hydrophobic groups in FBPIs (Xu et al. 2016). Thus, DH9/DH15-FBPI exhibited decreased emulsifying capacities compared DH0-FBPI (control) and DH4-FBPI. Similar results were reported by others that demonstrated undue hydrolysis would impair emulsifying power (Turgeon et al. 1992).

Collectively, enzymatic modification is a powerful tool for improving emulsifying properties of proteins by producing favorable MWs and physiochemical properties. However, the enzymatic reactions need to be carefully controlled to avoid excessive modifications that might result in opposite effects. Mild modifications with applying ross-linking by MTGase (MTG60-FBPI) and hydrolysis by Alcalase (DH4-FBPI) produced either comparable or better emulsifying capacities as compared to those of the native FBPI.

6.2.2 Effects of combination with CH

The binding of CH to FBPI (soluble FBPI-CH complex) induced lower surface charge and thus lower electrostatic repulsion in corresponding emulsions as compared to the native FBPI (study III). It would imply that forming a complex with CH did not improve the emulsifying capacity (Joshi et al. 2012). In addition, the introduction of hydrophilic CH to FBPI remarkably lowered the hydrophobicity of FBPI and potentially resulted in reconfigurations, which together might exert unfavorable effects on the emulsifying capacity. It has been established that the surface charge of protein molecules, together with hydrophobicity strongly influences their ability to lower interfacial tension (Magdassi and Kamyshny 1996). The interfacial activity of FBPI would be potentially impaired by CH binding because of reduced availability of the hydrophobic binding segments, as well as the smaller diffusion coefficient caused by the larger size (Jourdain et al. 2009). Therefore, it was expected that the soluble FBPI-CH complex would have lower ability to decrease the interfacial tension than FBPI. The ability of proteins to lower the interfacial tension at the oil-water interface is critical to form a stable P-O/W EM, (Mikkonen et al. 2019). However, similar levels of interfacial tension were found in FBPI and soluble FBPI-CH complex. One possible explanation was that more FBPI-CH complex was adsorbed to the interface, which consequently enhanced the surface activity (Jourdain et al. 2009).

In summary, FBPI exhibited more favorable physicochemical properties towards being a better emulsifier than the soluble FBPI-CH complex. In most cases of simple P-O/W EMs, the property of interfacial region is determined by proteins (emulsifiers) alone. However, considering the participation of CH, multiple factors such as physiochemical properties, layer structure, and location of FBPI and/or CH and their interactions should be taken into account when studying the interfacial layer properties.

6.3 Effects of FBPI modifications on interfacial layer

6.3.1 Protein adsorption

Protein adsorption fraction (F_{ads}, %) is an important interfacial layer property that affects emulsion stability. Emulsions with hydrolyzed FBPI had a higher surface coverage at the interfacial layer as shown by higher F_{ads} results (study II). According to Schroder et al. (2017), flexible peptides produced by hydrolysis could facilitate the migration to the oil-water interface, and thereby improve the protein adsorption. EMC and EML had much higher F_{ads} as compared to EMFB and EMN (study III). This could be explained by the favorable co-adsorption of CH with FBPI at pH 4.8 due to electrostatic interaction, as illustrated by Laplante et al. (2006). Meanwhile, a LBL or mixed-layer system is prone to trap more protein and/or polysaccharide, thereby increasing the protein adsorption (Yuan et al. 2013). The higher F_{ads} in EMC might at least partly contribute to the surface activity of the soluble FBPI-CH complex in EMC, as discussed in **chapter 6.2.2**.

6.3.2 Interfacial layer thickness

The interfacial layer thickness is another important factor affecting emulsion stability. MTG induced crosslinking at the amino acid side chains resulting in branched polymers and steric constraint (study I). As a result, a thick layer protruding from the surface of the oil droplet could be formed, which might induce increased emulsion stability (Kargar et al. 2012). This was also seen by increased emulsion particle size in the freshly made MTG-emulsions.

To better understand the effects of layer thickness on emulsion stability, the layer thickness was measured by using polystyrene latex beads (study III). The interfacial layer thickness of EMFB and EMN were similar as they were both emulsified primarily by FBPI. CH would not contribute to the interfacial layer of EMN as EMN was emulsified by FBPI before the addition of CH (Jourdain et al. 2009), and the emulsifying property of CH was far less than FBPI (Klinkesorn 2013). The notable difference in layer thickness between the monolayer systems (EMFB and EMN) and EMC/EML may be mainly induced by electrostatic binding of CH to FBPI. Chattopadhyay and Inamdar (2010) proposed that CH was present mostly in a loosened coil conformation rather than extended straight form even when fully dissolved in solutions. Thus, CH would tend to connect to and cover FBPI in a more steric form (thicker) rather than a flat form (thinner). Compared to EMC, the thicker layer in EML might be attributed to the LBL system in which CH was expected to sit on top of a ~ 19.1 nm preformed FBPI-layer, as schematically illustrated in **Figure 13B**. The distribution/assemble of FBPI and CH at the interfacial layer was further visualized by CLSM. It was worth noting

that, compared to the soluble FBPI-CH complex in EMC, FBPI appeared to form a denser inner layer around the oil droplet in EML. It was possible that FBPI had better emulsifying capacity and relatively smaller molecular size, which facilitated a more uniformed secondary layer by CH. On the other hand, the large CH might pose steric hindrance on FBPI and led to disorganized protein adsorption in EMC (Jourdain et al. 2009).

To the best of our knowledge, study III was the first report on visualizing CH and protein together in emulsions by using synthesized FITC-labeled CH. It should be pointed out that, as CH was colored by the FITC, the green color layer did not reflect the real thickness scale of CH compared to FBPI. That may explain why the green CH layer was seemingly thinner than the red FBPI layer, even though the main contribution of the interfacial thickness should be from CH as shown by the layer thickness measurement above.

6.3.3 Low-shear viscosity of interfacial layer

As demonstrated above, a thicker and less mobile interfacial network was generated by the electrostatic bonding of CH to FBPI in EML and EMC, which consequently led to a higher interfacial shear viscosity (Jourdain et al. 2009). The thickest, densest, and compact interfacial layer of EML corresponded to the highest low-shear viscosity.

6.4 Effects of FBPI modifications on physical stability of emulsions

6.4.1 Protein adsorption

The changes in protein adsorption/desorption at the oil-water interface reflect physical stability of emulsions. The level of F_{ads} in DH4 was maintained during storage, indicating physical stability of the emulsion containing mildly hydrolyzed FMPI (study II). By contrast, the levels of F_{ads} in DH9 and DH15 were dramatically decreased, even though the levels of initial protein adsorption were comparable or even higher than that in DH0. It has been proposed that extensive DH produced smaller peptides which were more readily desorbed as a result of higher net charge and dramatically reduced hydrophobicity (Schroder et al. 2017). In study III, the levels of F_{ads} in EMFB, EMN and EMC were maintained during the storage. Interestingly, the level of F_{ads} in EML was gradually increased. It was possible that more free aqueous FBPIs were gradually bonded to the CH which was coated outside the bilayer via electrostatic attraction. The high F_{ads} in EML might facilitate decreasing of the interfacial tension and thus further improve emulsion stability (Jourdain et al. 2009).

Adequate and stable amounts of protein at the interfacial phase is one of preconditions for sustaining emulsion stability. Therefore, DH0 and DH4 (study II) and EML (study III) showed better physical stability, which was also supported by changes in droplet size and Turbiscan as discussed below.

6.4.2 Droplet size

The change in droplet size was an another important index for evaluating physical stability of emulsions. Therefore, we monitored the particle size by Mastersizer and microscopy. Compared to MTG120 and MTG240 emulsions, MTG0 and MTG60 emulsions exhibited smaller droplet size and more homogenous distribution (study I). This might be attributed to their corresponding emulsifiers (MTG0/60-FBPI) which had better emulsifying properties as demonstrated before. On the other hand, extended MGT treatment (MTG120/240-FBPI) led to poorer physicochemical characteristics of emulsifiers. Similarly, DH0 and DH4 had smaller particle size and homogeneous and stable distribution over the storage time (study II). This might also be attributed to the better emulsifying properties of DH0-FBPI and DH4-FBPI. In contrast, the less favorable emulsifying properties of DH9-FBPI and DH15-FBPI produced less stable emulsions, as indicated by markedly increased droplet size.

EMFB and EML displayed a monomodal distribution and stable droplet size during storage, indicating better physical stability (study III). This might be at least partly explained by the more favorable emulsifying property of FBPI than that of the soluble FBPI-CH complex. In addition, the thicker interfacial layer of EML may also contribute to emulsion stability via steric stabilization. Although EMC had a relatively thicker interfacial layer that could prevent droplet flocculation by increasing the steric repulsion, such effects might be counteracted by the poorer emulsifying capacity of the complex, the disorganized/loose interfacial layer and the lowest zeta potential for emulsion.

Together, these results indicated that the emulsifying properties of a protein have a more important effect on the physical stability of the P-O/W EMs than steric factors.

6.4.3 Turbiscan and microscopy observation

Turbiscan and microscopy observations were applied to better characterize the phenomena of destabilization. Turbiscan revealed flocculation and coalescence in DH9 and DH15 (study II), as well as in EMC and EMN (study III), which was in accordance with larger fat globules shown by microscopy observation and the changes in droplet size. However, it was noticed that the bigger oil droplets in EMN observed via microscope and Turbiscan were not reflected by either $d_{3,2}$ or $d_{4,3}$. One possible reason for the discrepancy may be that the

Mastersizer measurement included a step of high-speed agitation that would break down the weak flocculation initially developed in EMN, while microscope observation and Turbiscan measurement only required standing or gentle stirring. Nevertheless, the relatively low stability in EMN agreed well with the decreasing F_{ads} during storage.

Overall, MTG60 (study I), DH0 and DH4 (study II), and EML (study III) showed better physical stabilities, as indicated by the changes in protein adsorption, droplet sizes, as well as Turbiscan and microscopy observation. This corresponds to the improved physiochemical characteristics of modified FBPIs and enhanced interfacial layers.

6.5 Effects of FBPI modifications on oxidative stability of emulsions

Oxidative stability is critical for the quality of emulsion-based foods, but it is underestimated when assessing either the emulsifying functionalities of plant proteins, or the stability of P-O/W EMs. Therefore, the lipid and protein oxidation were monitored during storage, and the association between physical stability and oxidative stability in emulsions prepared by modified FBPIs was further investigated. Moreover, increasing evidence suggests that oxidation products from either proteins or lipids can further expedite the oxidation in a reciprocal manner (Faustman et al. 2010). Thus, the focus was also on the interrelationship of lipid oxidation and protein oxidation and its influence on the overall oxidative stability of P-O/W EMs.

6.5.1 Lipid oxidation related to proteins

In all three studies, the progression of lipid oxidation took place in all emulsions, as indicated by increased formation of CDs and secondary lipid oxidative products. Lipid oxidation may occur rapidly in O/W emulsions due to their large surface area that facilitates interactions between lipids and water-soluble prooxidants (McClements 2007). At the end of storage, emulsions emulsified with MTG60-FBPI and MTG120-FBPI, but not with MTG240-FBPI, exhibited improved oxidative stability of lipids, as evidence by lower formation of CDs and hexanal compared with the control emulsion (study I). Similarly, Ma et al. (2012) reported that moderate MTG treatment on casein improved the oxidative stability in flaxseed oil emulsions while increased dose of MTG did not further improve the effects. The emulsions with DH4-FBPI (mild hydrolysis) exhibited the least lipid oxidation (study II). This was in line with previous findings showing that moderate hydrolysis could improve the emulsifying property (Schroder et al. 2017). Therefore, similarly to the effects on emulsifying properties of FBPIs, appropriate enzymatic modifications could improve the lipid oxidative stability.

EML emulsion, prepared according to a LBL system, exhibited the least productions of both primary and secondary oxidation, indicating the best lipid oxidative stability, compared with the single layer (EMFB and EMN) and mixed interfacial layer system (EMC) (study III).

To identify the most crucial factor for lipid oxidation in O/W emulsions is challenging, as the O/W emulsion is a complicated system that consists of not only lipid phase as bulk lipids, but also aqueous phase which contains both prooxidants and antioxidants, as well as the oil-water interface that impacts interactions between oil and water components (McClements and Decker 2000). Any triggering factor from either lipid phase, aqueous phase or interfacial phase can potentially influence the rate of lipid oxidation in O/W emulsions. For example, environmental factors (e.g. aqueous phase pH, ionic strength, and oxygen concentration), compositions related factors (e.g. fatty acid composition, degree of unsaturation, type and concentration of antioxidants and prooxidants), lipid droplet characteristics (e.g. particle size, concentration, and physical state), as well as emulsion droplet interfacial properties (e.g. thickness, charge and rheology) could all be involved in lipid oxidation in emulsions (Waraho et al. 2011). The designed emulsion models were prepared with modified FBPI without adding other components (studies I and II). Therefore, it was expected from the protein-interfacial layer and proteins in the aqueous phase to play profound roles on lipid oxidation.

The oil-water interface also has a major impact on the lipid oxidation by influencing the location and reactivity of prooxidative transition metals, lipid hydroperoxides, minor lipid components, free radical scavengers, and metal chelators (Waraho et al. 2011). In study I, MTG treatment induced cross-linking at the side chains of FBPIs, which created branched polymers and steric constraint. The majority of the peptide segments of the branched polymers may not directly contact with the interface. As a result, a thick interfacial layer would be presumably formed by the peptide segments protruding from the surface of the oil droplet. The physical properties of the interfacial layer such as the thickness and compactness would strongly influence oxidation kinetics by affecting the interaction between aqueous phase prooxidants and lipid cores. In emulsions, the lipid oxidation starts with decomposition of lipid hydroperoxides located at the droplet surface into free radicals promoted by transition metals (Nuchi et al. 2001). Then, lipid oxidation is initiated by oxygen transported into the oil phase via the interface (McClements and Decker 2000). Thus, a thicker and denser protein-structure between aqueous prooxidants and oil phase may limit lipid oxidation by acting as a physical barrier (McClements and Decker 2000; Ma et al. 2012).

To better understand the impact of interface thickness on oxidative stability, emulsions were formulated with different interfacial structures by combining chitosan with FBPI (study III). Then, the relationship between interfacial layer thickness/compactness and lipid oxidation was explored. The least lipid oxidation was found in EML which also had the thickest and cohesive/compact layer. This enhanced interfacial layer may slow down lipid oxidation by retarding the diffusion of oxygen and prooxidants, or the transition of metal ions. In addition, the thicker layer in EML improved physical stability by preventing droplet flocculation or coalescence via steric stabilization, which might also enhance the lipid oxidative stability. It needs to point out that layer thickness was not the solo determining factor for lipid oxidation. In EMC, which also had greater layer thickness, did not show improved lipid oxidative stability. One possible reason was the emulsifier-packing density at oil-water interface. The density of interface may impact lipid oxidation by influencing the diffusion of oxygen, free radicals, and prooxidants through the interfacial layer (Villiere et al. 2005). In the interface of EMC, the large CH might pose steric hindrance on FBPI and lead to disorganized and un-cohesive interface. As a result, the interface might contain much more pores that facilitated the diffusion of prooxidants, and consequently enhanced lipid oxidation.

Besides the thickness and compactness, the charge of interface induced by interfacial proteins is another critical factor to consider when evaluating oxidative stability of emulsions. Since metal ions promote lipid oxidation, the interface charges might speed up or slow down lipid oxidation by attracting or repelling cationic metals ions in the aqueous phase (Waraho et al. 2011). Extensive hydrolysis (DHs of 9% and 15%) produced increased net negative surface charges of FBPI that might attract pro-oxidative cationic metal ions, and thereby led to decreased stability in lipid oxidation (study II). This was in agreement with previous studies showing that emulsions prepared with anionic surfactants expedited lipid oxidation due to the electrostatic attraction of cationic transition metals whereas cationic surfactants decreased oxidation rates by electrostatically repelling metals (Mancuso et al. 2000; Silvestre et al. 2000; Boon et al. 2008).

The emulsifying property of a protein assembling at interface is also closely associated with lipid oxidation. DH4-FBPI displayed better emulsifying performance and correspondingly produced an emulsion with greater lipid stability (study II). The limited hydrolysis produced a suitable lower MW with a more flexible peptide structure that allowed for greater mobility at the oil-water interface and better penetration into oil. The hydrolysis also generated moderately increased surface charges that produced increased repulsive electrostatic force, and increased hydrophobicity for better anchoring at the interface. Hebishy et al. (2017) also

demonstrated that the ability of whey protein isolates to inhibit lipid oxidation in O/W emulsions was due to its satisfying emulsifying property. Conversely, unfavorable emulsifying activity might predict increased lipid oxidation. For example, the soluble FBPI-CH complex exhibited poorer emulsifying capacity due to lower surface hydrophobicity and surface charge, which might partly explain the unsatisfied lipid oxidation.

Other than the proteins responsible for emulsification located at the interface, the free proteins in the aqueous phase might inhibit lipid oxidation by chelating metal ions, converting hydroperoxides, or quenching free radicals (Pokorny et al. 2001; Elias et al. 2008). It has been demonstrated that the metal chelating activity and free radical scavenging of native proteins can be increased by moderate enzymatic hydrolysis which exposes antioxidant amino acid residues buried in the protein interior (Elias et al. 2008; Sarmadi and Ismail 2010) . Moderate hydrolysis with Alcalase (DH of 4%) improved the ability of FBPI for maintaining stability toward lipid oxidation in O/W emulsions, which might be attributed to increased number of exposed antioxidant amino acid residues (study II).

Lipid oxidation is also affected by other ingredients in the emulsion matrix. For example, in the EMN displayed expedited lipid oxidation which might be attributed to the free CH in the aqueous phase (study III). CH was expected to inhibit lipid oxidation due to its ability to chelate metals ions (Chattopadhyay and Inamdar 2010). However, the metal ions might become more reactive after chelation by CH. The participation of CH could increase the solubility or convert the ions to their more reactive states, resulting in more decomposition of the CDs and conversion to secondary lipid oxidation products (Chen et al. 2011a). As Berton-Carabin et al. (2014) proposed, the lipid oxidation products in aqueous phase may modify some characteristics of the interfacial layer, which may favor the migration of reactive species to the oxidizable substrates.

Collectively, appropriate enzymatic modifications by either MTG (MTG60) or Alcalase (DH4), as well as combination with CH via a LBL model (EML) could inhibit lipid oxidation in O/W emulsions. The inhibition is not only attributed to improved emulsifying activities of FBPI and interfacial properties, but also explained by protective effects by proteins in the aqueous and interface phase.

6.5.2 Protein oxidation related to lipids

Protein oxidation is closely associated with lipid oxidation. More importantly, it can decrease food quality and therefore should not be underestimated. Protein oxidation was determined by measuring the loss of Trp and sulfhydryl, and formation of carbonyls. Protein oxidation

is affected by various external factors (e.g. temperature, pH, partial oxygen pressure, lipid oxidation) and internal factors (e.g. amino acids composition and physical structure of the protein) (Renerre 2000).

Proteins may protect other food components such as lipids from oxidation at the expense of themselves. Such effects have been mainly attributed to the capacity to chelate metal ions by Trp, His, Glu, Asp, and phosphorylated Ser and Thr, as well as the ability to scavenge free radicals by aromatic and sulfur-containing amino acids (Saiga et al. 2003; Ueda et al. 2003; Lund et al. 2011). In addition, proteins could convert hydroperoxides into inactive hydroxylic derivatives, thus disrupting lipid oxidation (Pokorny et al. 2001). The effect of a protein to protect lipid from oxidation not only depends on its amino acids composition, but also is affected by the three-dimensional structure. The protective effect may be limited by the tertiary structure because many amino acids with antioxidant potential are buried within the protein core where they are inaccessible to prooxidants (Elias et al. 2008). Some protein modifications can expose amino acids with free radical scavenging activities which are originally located in the interior; consequently, the overall protective effect of proteins are enhanced. MTG treatment for 120 and 240 min significantly increased the surface hydrophobicity of FBPI (study I). This indicated more exposure of nonpolar amino acids previously embedded inside the protein structure. The shift of λ_{max} suggested that Trp residues in MTG-FBPIs emulsions were exposed more to the aqueous polar environment (Marcuse and Fredriksson 1968). In addition, MTG treatment produced a higher degree of unfolding with increased exposure of oxidizable hydrophobic residues to the solvent, resulting in a less compact structure of protein (Moro et al. 2011). Similarly, heat treatment (70 to 130 ˚C for up to 30 min) increased the antioxidant activity of skim milk by exposing sulfhydryl groups of Cys (Taylor and Richardson 1980).

Considering that the emulsions were formulated with oil, water, and proteins, lipid oxidation was expected to be an indispensable factor that influences protein oxidation. It has been proposed that the primary and secondary products derived from unsaturated fatty acids might promote protein oxidation (Lund et al. 2011). For example, peroxyl radicals, formed during lipid oxidation, can abstract hydrogen atoms from protein molecules and lead to a radical mediated chain reaction similar to that of lipid oxidation (Stadtman and Levine 2003). In addition, hydroperoxides, surface-active compounds that are primarily produced during lipid oxidation, can easily migrate to the interfacial layer where proteins are located and thereby promote protein oxidation (Schaich 2013). DH9 and DH15 showed greater protein oxidation and lipid oxidation than DH0 and DH4 (study II). Lipid oxidation appeared to proceed before

protein oxidation, as hexanal was above baseline on day 1 while the protein oxidation markers were above baseline until day 4. The rapid protein oxidation may be the result of generation of reactive species during lipid oxidation which would cause the oxidative damage of proteins. At the later stage of storage, both lipid oxidation and protein oxidation occurred extensively. It has been reported that protein and lipid oxidation could proceed via free radical transfers between proteins and lipids, propagating the co-oxidation (Berton et al. 2012). Previous studies showed that adsorbed proteins were more prone to oxidative modifications than continuous phase proteins because adsorbed proteins were at the interface and were more prone to lipid radical-initiated oxidation and protein radicals triggered oxidation (Rampon et al. 2001; Gürbüz 2018). However, the results showed that the percentage of protein oxidation in the aqueous phase was more pronounced than that in the interface. More than 70% of the proteins or protein hydrolysates was unadsorbed and distributed in the aqueous phase (study II). This was representative in most P-O/W EMs, where the majority of the protein was presumably unadsorbed and present in the aqueous phase as a result of a higher amount than actual amounts required to cover the interfacial surface. The contribution of unadsorbed protein to oxidative stability seems to be dominant because they are abundant and more exposed to prooxidants such as oxygen or metal ions. Moreover, extensive hydrolysis (DHs of 9% and 15%) led to lower emulsifying capacity of FBPI, and consequently poorer emulsion stability, which might further exacerbate the protein oxidation.

As shown in lipid oxidation, interfacial layer properties had a major impact on lipid oxidation. We further examined how interfacial layer affected protein oxidation in study III. Both EML and EMC were formulated with FBPI and CH with greater interfacial layer thickness than EMFB and EMN, but they had different protein oxidative stability. EML had greater protein oxidative stability than EMFB and EMN, although the latter two emulsions had stronger repulsion against transition metals due to the higher cationic charge of emulsion droplets. In EML, a thicker and cohesive/compact LBL by FBPI and CH was formed at the interface. As discussed in lipid oxidation, the thicker interface could physically inhibit the ability of metal ions to access lipid hydroperoxides and consequently retard lipid oxidation in O/W emulsions. This mechanism might also apply to protective effects of the LBL system on protein oxidation. Compared with EML, EMC exhibited much higher protein oxidation indicated by the loss of Trp and the generation of carbonyl, although both had thicker interfacial layers due to CH participation. In EMC, CH was introduced as a soluble complex with FBPI for emulsification. The large CH might pose steric hindrance on FBPI and led to

disorganized protein adsorption in EMC. Thus, FBPI was not well protected by the CH layer as shown by CLSM images. In comparison, FBPI in EML formed a denser inner lay around the oil droplet due to its better emulsifying capacity and relatively smaller molecular size, which facilitated a more uniformed secondary layer by CH.

Interestingly, lower loss of sulfhydryl concentration was observed in both EML and EMC (study III). This might be tied to the solubility of presented CH rather than the position of CH. The Ellman's reaction was performed at its optimal pH of around 8.0 where CH was partly insoluble. Some FBPIs may be trapped inside the insoluble CH and removed in the washing procedure during the measurement. As shown by Bravo-Osuna et al. (2007), the lower the sample concentration was, the higher the amount of sulfhydryls determined per gram of protein was. This was well in agreement with our finding that the sulfhydryl concentration was increased, along with the decreased sample concentration as precipitates were removed before spectrophotometric determination. Although the sulfhydryl results in this study was of limited reflection to protein oxidation in the presence of CH, it still demonstrated the potential protection of sulfhydryl against oxidation by CH via steric accessibility.

Overall, although proteins in both aqueous and interface phase may protect against lipid oxidation via different mechanisms, the potential damages on food quality and nutrition by protein oxidation should not be neglected. We have observed different levels of protein oxidation in all three studies, which further emphasizes the importance of monitoring protein oxidation in emulsions. We also showed that either enzymatic modification or physical association with CH could affect the development protein oxidation in P-O/W EMs by affecting the protein configuration and interfacial layer properties.

7 Conclusions

Faba bean is a promising plant protein source due to its high protein content, easy cultivation, and various functional properties for food production. However, some functional properties of FBPI such as emulsifying properties are limited by its intrinsic physiochemical properties. Thus, in this study the FBPI was modified by enzymatic treatments and examined in combination with CH in order to improve the emulsifying property and to enhance the physical and oxidative stability of O/W emulsions. The potential interrelation among physical stability, lipid oxidative and protein oxidative stabilities were explored.

This study demonstrated that moderate enzymatic treatments by either MTG or Alcalase could improve the emulsifying functionality of FBPI. Specifically, MTG treatment for 60 min makes FBPI a potential emulsifier resulting in maintained physical stability with improved lipid oxidative stability in emulsions. Moderate Alcalase hydrolysis (DH of 4%) of FBPI improves both physical and oxidative stability of O/W emulsions. The FBPI hydrolysates exhibited flexible peptide structures, increased surface charge, and hydrophobicity that favored emulsifying capacity. However, it needs to be emphasized that prolonged enzymatic treatment (MTG > 60 min or Alcalase > 4%) should be avoided, as they might negatively affect the emulsifying activity of FBPI and thus the stability of the emulsions.

Besides enzymatic treatments, physical modification by combining FBPI with CH via electrosteric interaction could improve O/W emulsion stability as well. In this study, different ways of assembling of FBPI and CH at the interface and the aqueous phase were examined. When added as a secondary layer of the FBPI-emulsified emulsion, CH could improve the physical and oxidative stability. This improvement was due to the LBL interfacial structure, which increased the interfacial layer thickness/compactness and maintained the interfacial protein adsorption. However, when CH was first formed a soluble complex with FBPI and then used as emulsifier, or added as free non-interactive form in emulsions, it had unfavorable effects on the emulsion stability.

Last but not least, a strong interrelationship among physical stability, lipid oxidative and protein oxidative stabilities was revealed: improved physical stability is associated with increased oxidative stability and proteins may act as antioxidants to reduce lipid oxidation in emulsions. Considering the potential damage on food quality and nutrition by protein oxidation, it is critical to take all three aspects into consideration when assessing the stability of protein-contained emulsions.

These findings expand the current knowledge on utilization of FBPI in food matrix and provide a feasible, safe, and economic way for improving physical and oxidative stability of O/W emulsions. It is anticipated that this work will be of benefit for the utilization of faba bean in food industry.

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